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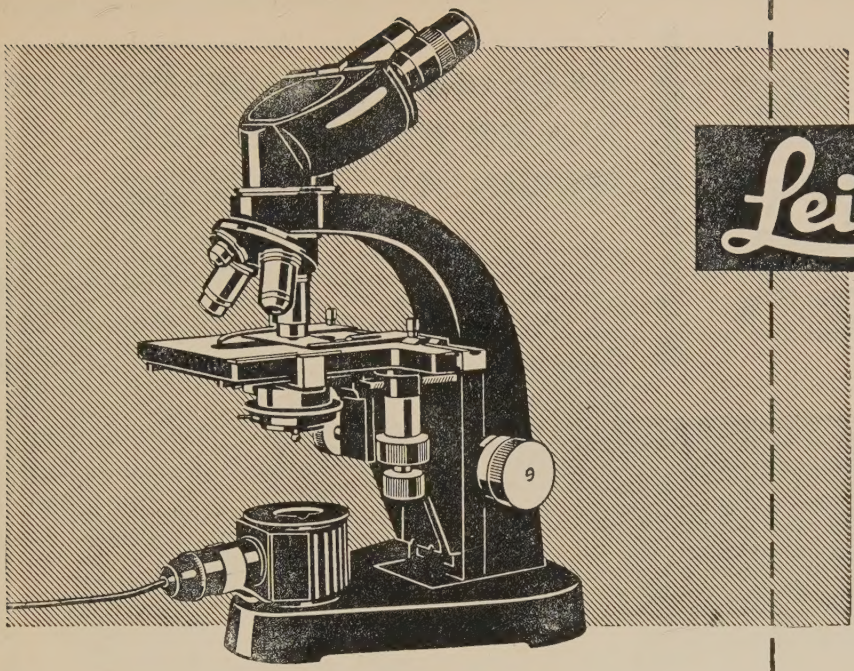

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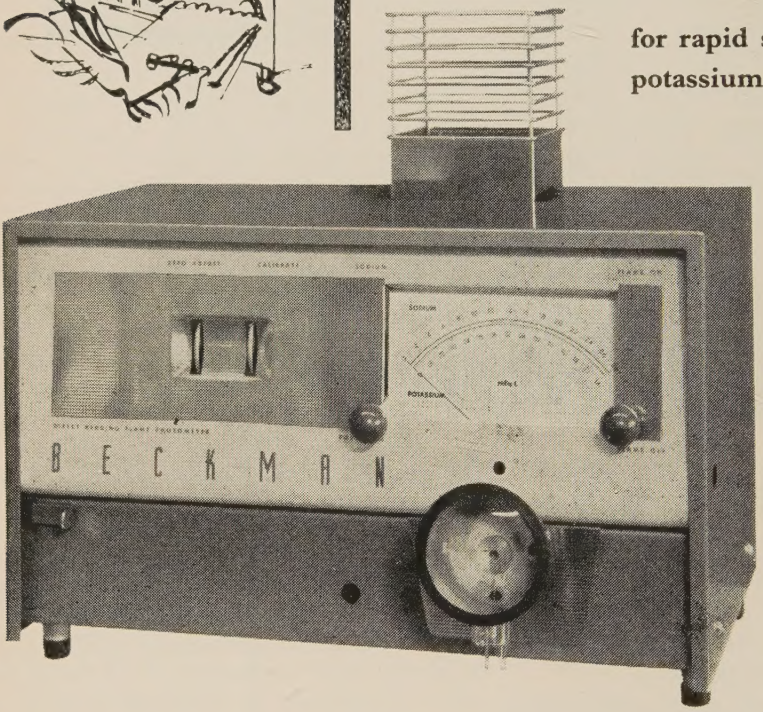

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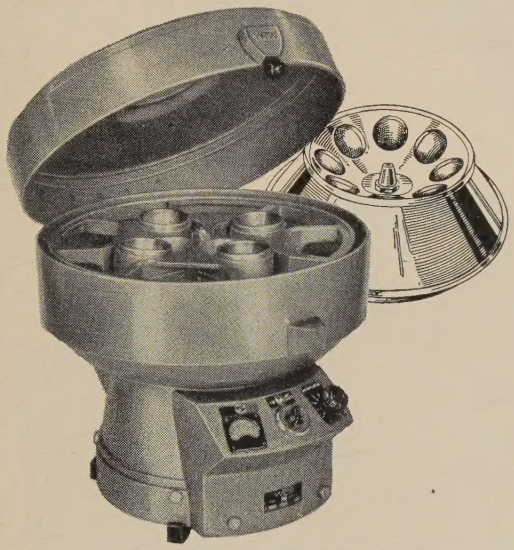

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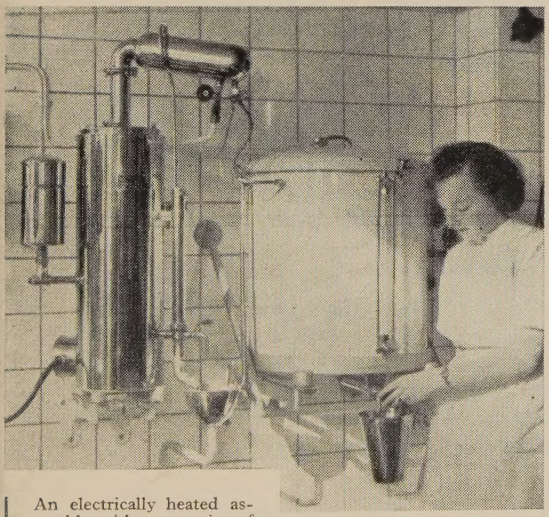

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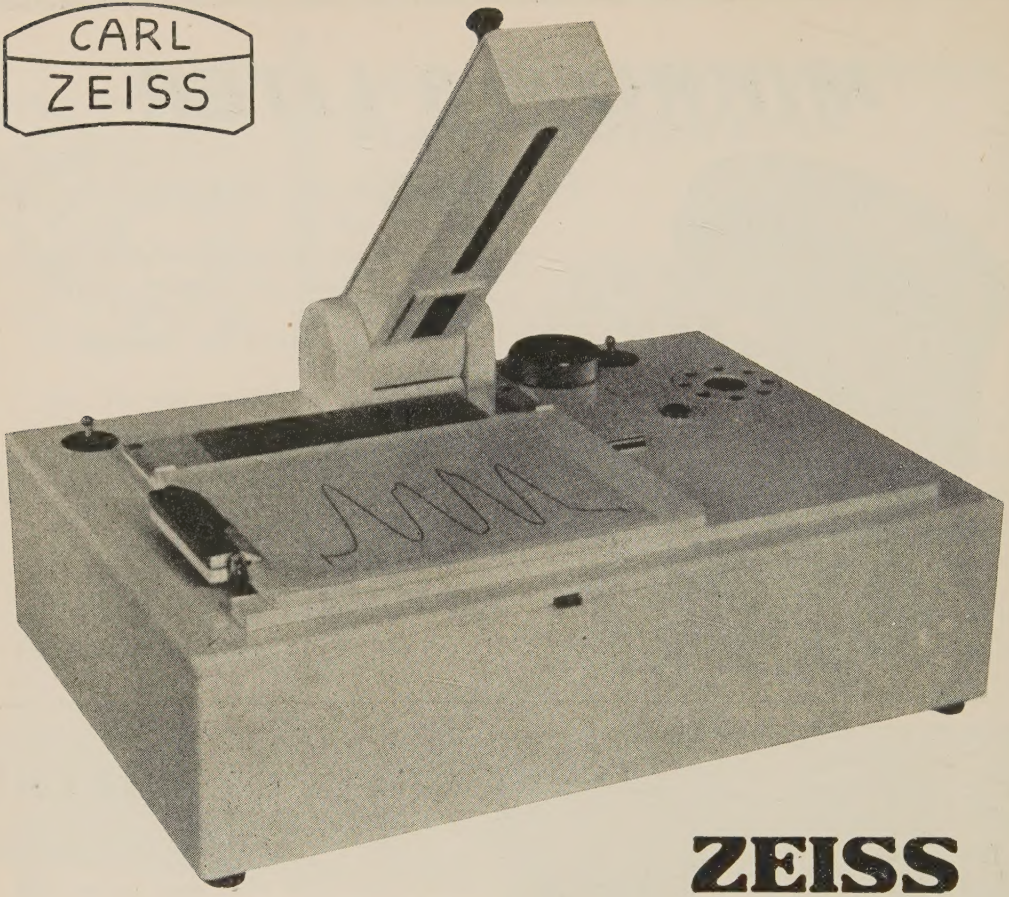

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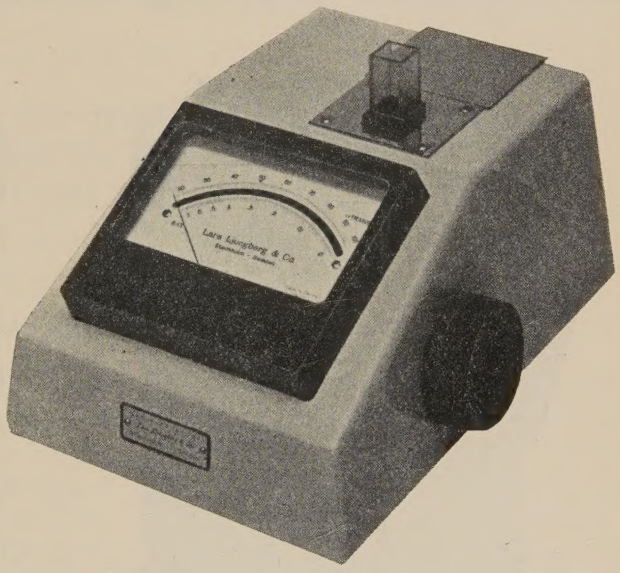

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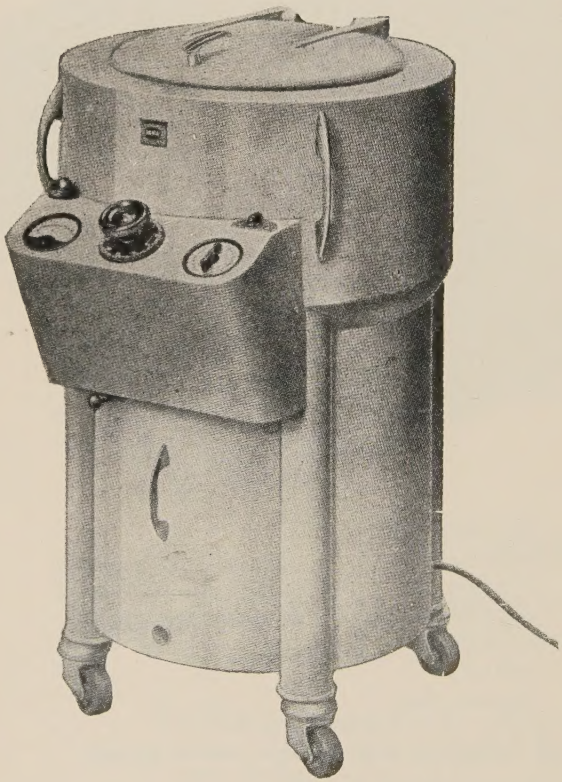

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INVESTIGATIONS ON THE INFLUENCE OF DIET ON THE QUANTITY AND COMPOSITION OF INTESTINAL GAS IN HUMANS

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From The Medical Service of the Royal Norwegian Air Force, Oslo, Norway

(Received for publication October 6, 1955)

The purpose of this investigation has been to determine the significance of diet for the quantity and composition of intestinal gas. It was undertaken with regard for flying personnel, whose physical comfort is of primary importance. Zimmermann (1909), Donaldson (1922), Alvarez (1924) and Percy & Allen (1927) have shown that even slight increase of pressure, from 30—60 mm Hg, in rectal gas, may lead to various vegetative signs of discomfort such as headache, dizziness, slight mental confusion, reduced ability to concentrate and slight retinal edema with reduced accomodation.

A definite volume of gas under standard conditions (0° and 760 mm Hg) will, under otherwise similar conditions, expand when the pressure is decreased. At 30,000 feet the volume is 4 times and at 40,000 feet 7.6 times as great as at the sea level (Armstrong, 1939).

During slow ascent the excess air will be expelled by mouth or by rectum, but during rapid changes of altitude this adaptation mechanism is inadequate. It would, therefore, be advantageous if the amount of intestinal gas cuold be decreased by means of diet.

There are two main sources of these gases:

1. *Swallowed atmospheric air*

All swallowing involves the swallowing of air. Alvarez (1939) has determined approximately the magnitude of this quantity. By means of fluoroscopy he found that the volume of air swallowed while drinking a glass of milk was about three times the volume of the milk.

2. *Decomposition of intestinal contents*

Decay plays a relatively insignificant part, but the fermentation of carbohydrates

produces large quantities of carbon dioxide, methane and hydrogen.

The quantity and composition of the gases is altered during intestinal passage as a result of diffusion. Blair, Dern & Bates (1947) estimate this gas turnover at 10 liters per day.

Intestinal gas in humans can be analysed only as rectal gas. Since the factors which influence diffusion are very difficult to observe, the rectal gases afford only a rough approximation of what has taken place in the upper regions of the intestines. Schoen(1925) demonstrated that the muscle tone and blood content of the intestinal wall are significant for diffusion. Schmidt & Strasburger (1901) showed that the rate of passage is also important as they found less rectal gas in constipated subjects.

The propoortion of the various gases will vary according to the relative rates of diffusion determined by Benedict, McIver & Redfield (1926) at N_2=1,CH_4=3, H_2=7, O_2=13, H_2S=69 and CO_2=160. Oxygen will not follow the laws of diffusion because it becomes bound to hemoglobin, while the absolute volume of nitrogen will remain relatively constant.

As regards the gases derived from fermentation, the carbon dioxide will rapidly reach an equilibrium with the tissue content at about 6.0 per cent as shown by Yllpö (1916) and Pogrund and Steggarda (1948). A high CO_2 content in rectal gas will be found when production is high and intestinal passage rapid. Hydrogen and methane should theoretically follow each other.

EARLIER INVESTIGATIONS ON THE QUANTITY AND COMPOSITION OF INTESTINAL GAS

Most of these are observations over a single day on one or more persons. The amounts vary from 0.280 l/day to 2.0 l/day (Blair *et al.*, 1947; Fries, 1906; Beazell & Ivy 1941; Kirk & Möller, 1946).

The composition of the gas varies from CO_2 9.0—25.4 per cent, O_2 0.0—5.6 per cent, CH_4 0.4—30.0 per cent and H_2 0.0—44.0 per cent. N_2 is always calculated as the remainder.

EARLIER INVESTIGATIONS ON THE INFLUENCE OF DIET

Blair *et al.* (1947) have shown that the amount of flatus on a fat diet of soya beans went up to 3.7 l per day while on a diet rich in carbohydrates it was 0.6 l per day. Kaunitz & Leiner (1936) compared flatulence on an ordinary diet with a vegetarian diet and found that the cellulose-rich diet gave the lowest values. Kirk (1947) found a 50 per cent increase in quantity on a diet which included Brussels sprouts compared with a standard diet. It was mainly the nitrogen and oxygen which were increased and he believes that perstaltic stimulation was the cause.

Certain foodstuffs have a tendency to give subjective symptoms of meteorism and dyspeptic discomfort (Hove, 1946). The subjective phenomena appear so rapidly after ingestion that they cannot be due to gas production. Alvarez (1939) believes this to be a slight food allergy, while Kirk (1947) claims that all ingestion of food results in peristalsis which gives the impression of gas tension.

PERSONAL INVESIGATIONS

Earlier investigations have given highly divergent results, partly due to differences in methods of collecting, but probably mostly because they included only one or a few days of observation for the individual experimental subjects. There is also little agreement as to the specific effect of various foodstuffs on the quantity of intestinal gas. For this reason an experimental series was planned continuously over a longer period. A basic diet with litttle roughage was planned as a starting point, and the individual foodstuffs chosen were given additionally, slightly in excess of what would be included in an ordinary diet.

METHODS

An apparatus was used for collecting the gas which was practically identical with Kirk's (1947). The total of dead space for the probe, rubber tubing and washing bottle was determined for each apparatus and the values found for intestinal gas were corrected for atmospheric air in the dead space.

For analysis of the gases they were transferred to a Hempel pipette filled with the same calcium chloride solution (sp. wt. 1.40) as in the bottles.

The analyses were performed immediately after each collection day and the contents of oxygen, hydrogen, methane and carbon dioxide were determined according to methods of Peters & Van Slyke (1932). All of the gases were brought to standard conditions 0° C and 760 mm Hg.

The content of the inert gases was not determined — they are included in the nitrogen values. The contents of hydrogen sulfide, mercaptans and ammonia were not determined either. Kirk (1947) found that these values were so small as to be insignificant in this type of investigation.

The nitrogen content is calculated as the remainder.

Choice of experimental subjects

The experiments were carried out in the women's department of Dikemark Hospital. Three schizophrenic demented women were selected, in the table denoted as persons Nos. 1, 2 and 3. Their respective ages were 36, 44 and 51 years. The duration of their disease was in all cases more than seven years and they exhibited a relatively constant pathological state.

It was necessary to perform the investigations on patients who were confined to their beds so that their freedom was not infringed and they also had to be indifferent to the experimental procedure. If the rectal probe caused irritation, the individual had to be excluded. They had to be fairly stabile and quiet, willing to eat and, as far as could be judged clinically, to exhibit such constant vegetative conditions as possible (judged by pulse, blood pressure, temperature, skin color, dermographism and pupillar reaction).

Experimental procedure

The daily collection period was 10 hours, from 0730 to 1730 o'clock. Meals were served at 0730, 1100 and 1800. The last meal was always after the collection period. A rest period in the middle of the day could not be included because of the hospital routine.

The gases were collected the first four days of each week, while the last three days of the week were rest days out of consideration for the patients. Each collection period covered eight days and extended over 2 calendar weeks. On the rest days which fell in the middle of a collection period the same diet was administered as during that period, and in the three days which followed a collection period the diet to be tested in the next period was administered. Thus the experimental subjects ate the diet to be tested for 14 days and startet three days before collection for that period was begun.

The diet

The starting point was a standard diet containing as little cellulose as possible consisting of milk, sugar, raw eggs with the addition of vitamins. The caloric content was 2500.

This diet was divided into three equal portions administered as the three daily meals and was given for a relatively long period before the actual experiment was begun. In the table this period is called Basic Diet A. Its length varies for the three experimental persons because it took some time to find three patients who were sufficiently stabile to begin the experiments.

The basic diet was administered for a period in the middle of the experiments as a control. In the table this period is called Basic Diet B.

The individual foodstuffs selected for investigation were divided into two equal portions and administered with the first two daily meals at 0700 and 1100 hours.

The foodstuffs investigated were:

Carrots, raw, grated	300 g
Cabbage, white, boiled	300 g
Potatoes, peeled, boiled	300 g
Peas, green, dried	150 g
soaked for 24 hrs, then boiled.	
Wholemeal bread	300 g
White bread	300 g
Meat, lean — veal, boiled	300 g
Meat, fat — mutton, roasted	300 g
Fish, cod, boiled	300 g

Two diets were compiled:

Diet I: Lean fish, 300 g, white bread 200 g, potatoes 200 g, butter 50 g, 1 egg, 1/2 liter melk.

Diet II: Fat meat 200 g, wholemeal bread 200 g, potatoes 200 g, butter 50 g, dried peas 50 g, 1 egg, 1/2 literm ilk.

In these periods the basic diet was dropped.

RESULTS AND DISCUSSION

The results are presented in the Table.

The figures in the table are the arithmetic means for the individual collection periods with the standard error of the means.

Due to various accidents, all of the collection periods did not cover eight days. As appears from the table, the range is rather wide both for quantity and composition. Compared with Kirk's (1947) investigations where the values represent the averages for 20 and 25 individuals, the range in the present investigations, judged by the standard error, is less than half that of Kirk. This is in good agreement with the values found by Blair *et al.* (1947) and is dependent on the considerable individual variation. It is further confirmed by a comparison between the three experimental individuals who, on the whole, stay close to individual levels throughout all of the collection periods.

The values for volume, calculated as volume per day, are about 250—600 ml/day. The volume of flatus at night is considerably less than during the day. The night volume was measured in two experimental individuals and gave 100 ml for both, while the corresponding volumes during the day were 380 and 290 ml.

The daily volume is somewhat higher than that found by Blair *et al.* (1947) on their average diet, but considerably lower than Kirk's (1947) figures. These, however, are averages of single collections from a number of experimental individuals.

The carbon dioxide values agree well with tissue content and the oxygen values are all low. This indicates slow intestinal passage in all the experimental individuals. Schizophrenic patients usually have sluggish intestinal passage, but according to Bleuler (1937) there is no reason to believe that the other functions of the colon are altered

Table.

The influence of various foodstuffs on the volume and composition of rectal gas.

	Individual – Diet	n	ml/min ± Δ	Per cent CO_2 ± Δ	Per cent O_2 ± Δ	Per cent CH_4 ± Δ	Per cent H_2 ± Δ	Per cent N_2 ± Δ
	Basic Diet A	24	0.68±0.078	5.2±0.326	0.6±0.122	0.4±0.102	2.3±0.531	91.5±0.714
	Basic Diet B	6	0.49±0.045	5.8±0.408	0.9±0.326	0.6±0.326	2.0±0.978	90.7±1.020
	Basic Diet+Raw carrots	6	0.72±0.045	6.8±0.408	0.7±0.163	0.2±0.122	0.4±0.285	91.9±0.449
	– +Cabbage	7	0.56±0.076	7.0±0.113	0.7±0.152	0.5±0.265	0.8±0.379	91.0±0.491
	– +Potatoes	8	0.55±0.057	6.0±0.106	1.5±0.248	1.5±0.389	1.1±0.176	89.9±0.425
	– +Peas	8	0.57±0.078	7.1±0.459	1.6±0.389	2.0±0.530	3.7±1.560	85.6±1.560
1.	– +Wholemeal bread	7	0.66±0.030	6.8±0.226	0.8±0.152	1.3±0.304	2.6±0.304	88.5±0.566
	– +White bread	8	0.69±0.102	7.8±0.248	1.0±0.354	0.5±0.318	3.4±1.318	87.3±1.698
	– +Meat, lean.	6	0.42±0.077	6.0±0.204	0.8±0.204	0.1±0.081	0.6±0.488	92.5±0.570
	– +Meat, fat	8	0.55±0.057	6.5±0.283	1.0±0.176	0.7±0.142	4.0±0.634	87.8±0.565
	– +Fish	8	0.42±0.081	5.4±0.320	1.8±0.389	0.4±0.106	4.0±1.170	88.4±1.200
	Diet I	6	0.65±0.065	6.2±0.244	1.2±0.122	0.5±0.082	1.6±0.530	90.5±0.612
	Diet II	8	0.71±0.085	7.2±0.320	1.4±0.389	2.6±1.450	3.1±0.778	85.7±1.380
	Basic Diet A	12	0.34±0.038	4.9±0.462	0.8±0.173	3.8±1.619	0.2±0.082	90.0±0.819
	Basic Diet B	6	0.39±0.032	5.5±0.205	1.7±0.410	0.3±0.205	0.3±0.082	92.2±0.327
	Basic Diet+Raw carrots	6	0.30±0.028	5.7±0.410	1.4±0.327	0.1±0.082	0.0±0.0	92.8±0.327
	– +Cabbage	6	0.32±0.069	5.2±0.327	1.3±0.410	0.1±0.082	0.0±0.0	93.4±0.368
	– +Potatoes	8	0.22±0.035	5.0±0.247	1.0±0.167	1.0±0.389	1.5±0.637	91.5±0.566
	– +Peas	8	0.38±0.060	7.1±0.707	0.8±0.142	4.2±1.415	1.5±0.460	86.4±1.483
2.	– +Wholemeal bread	7	0.26±0.026	5.3±0.265	1.9±0.340	3.7±2.190	1.2±0.492	87.9±2.620
	– +White bread	7	0.21±0.022	5.2±0.265	2.2±0.529	0.0±0.0	0.0±0.0	92.6±0.492
	– +Meat, lean	6	0.20±0.033	5.0±0.205	1.5±0.246	0.05±0.041	0.06±0.08	93.4±0.410
	– +Meat, fat	8	0.23±0.025	5.8±0.424	2.6±0.495	0.9±0.247	1.8±0.635	88.9±0.813
	– +Fish	7	0.31±0.056	5.3±0.265	1.5±0.303	0.0±0.0	0.0±0.0	93.2±0.227
	Diet I	5	0.30±0.045	6.2±0.375	1.1±0.179	0.2±0.179	0.3±0.179	92.2±0.760
	Diet II	8	0.66±0.088	7.0±0.328	1.0±0.283	10.6±1.801	1.4±0.247	80.0±1.839
	Basic Diet A	19	0.41±0.046	5.2±0.367	0.6±0.138	0.4±0.114	2.3±0.597	91.5±0.804
	Basic Diet B	5	0.40±0.062	5.4±0.226	0.7±0.226	0.6±0.179	3.0±1.790	90.3±1.790
	Basic Diet+Raw carrots	5	0.58±0.044	5.9±0.358	1.4±0.314	0.3±0.226	0.2±0.179	92.2±0.314
	– +Cabbage	5	0.40±0.028	5.2±0.278	0.8±0.179	0.6±0.314	0.6±0.179	92.4±0.670
	– +Potatoes	7	0.32±0.038	5.5±0.453	0.9±0.265	1.5±0.717	1.3±0.795	90.8±1.548
	– +Peas	6	0.54±0.037	6.2±0.245	1.8±0.326	1.7±0.326	1.3±0.449	89.0±0.778
3.	– +Wholemeal bread	2	0.38±0.064	5.4±0.565	1.5±0.442	1.4±0.354	3.4±1.554	88.4±0.920
	– +White bread	6	0.35±0.077	6.2±0.531	1.5±0.286	0.4±0.163	2.3±0.733	89.6±0.856
	– +Meat, lean	5	0.24±0.054	4.3±0.278	1.3±0.314	0.0±0.0	0.0±0.0	94.4±0.493
	– +Meat, fat	7	0.33±0.068	4.9±0.303	3.1±0.491	0.7±0.265	1.6±0.377	89.7±0.718
	– +Fish	3	0.19±0.040	5.3±0.578	1.1±0.231	0.1±0.115	0.2±0.231	93.3±0.923
	Diet I	5	0.65±0.044	7.0±0.314	1.9±0.089	0.3±0.089	1.0±0.179	89.8±0.718
	Diet II	7	0.55±0.109	6.2±0.377	2.1±0.491	1.4±0.377	3.2±1.058	87.1±1.207

n = number of collection days. Δ = standard error of mean value.

or that fermentation processes are anything but normal. The values for hydrogen and methane, however, should be lower in flatus from individuals with slow intestinal passage because of the increased opportunity for diffusion. The table shows that this is the case. The high values for the standard errors of the means for hydrogen and methane, however, show that there are some high individual values. These occur irregularly among the individual collection periods and have no relation with external fac-

tors such as the beginning or end of a collection period, tendency to diarrhea or the like.

No satisfactory explanation of these high, single values is afforded by the present experiments. There are probably vegetative factors which influence the rate of diffusion. McLester (1939) claims that flatus production is more a vegetative reaction than a mechanical production of gas, and Ingersoll & Jones (1946), by electrostimulation, have demonstrated centra in the neopallium which regulate colon function.

It is difficult to evaluate the influence of individual factors in the diet on the quantity and composition of rectal gas. The starting point was a basic diet which should theoretically have given the smallest quantities of gas because of its low cellulose content. The table shows that all three experimental individuals have periods where both the amount and per cent of fermentation gases are lower than on the basic diet. This shows that the foodstuffs chosen have had no definite influence on the quantity of gas in the rectum at the basic diet was constant and the various foodstuffs were given additionally.

This is further confirmed by a comparison between basic diet periods A and B for experimental individuals No. 1. The diets were identical and the difference must therefore be an expression of a variation which cannot be due to the special foodstuffs administered in the other periods. The difference is statistically significant at the 5 per cent level.

Such a difference between two identical periods renders an evaluation of other differences, which should be the result of diet, highly problematic. For this reason, and because the range is so wide, a more detailed statistical evaluation is not indicated.

The only indication of any effect of dietary factors may be found in the figures for Diet II which show the same tendency in all three experimental persons toward higher values both for quantity and for fermentation gases. This diet is characterized by a high fat content and a moderate quantity of vegetables. Blair *et al.* (1947) similarly found that the fatty soybean diet gave very high values for intestinal gas. It must be assumed that this diet causes increased peristalsis as it yields high values evenly distributed throughout the entire collection period both for volume, oxygen, carbon dioxide, hydrogen and methane. Because it is the fermentation gases which exhibit the relatively greatest and most evenly distributed increase, it must be assumed that fat content can influence the diffusion rate, for example by altering the surface permeability. Vegetables alone have no marked influence on either the volume or composition of the gas. This is in agreement with Kaunitz & Leiner's (1936) finding of the lowest values on an exclusively vegetarian diet.

CONCLUSION

The present investigation demonstrates that experiments of this nature which are carried out with the same experimental subjects over a longer period yield more stabile results than single experiments.

The quantity and composition of rectal gas, however, depends on so many factors beyond control that statistically reliable

onclusions cannot be drawn. The individual oodstuffs tested seem to have little or no lefinite influence on the rectal gas.

There is, however, an indication that a liet containing vegetables and an abundance of fat tends to give an increased volume of gas. This increase is probably greater than what would be expected as the result of ncreased peristalsis alone.

SUMMARY

To investigate the influence of certain oodstuffs on the production of intestinal as, three experimental individuals were dministered a liquid, cellulose-free diet. This should theoretically afford least possible material for gas production in the ntestine.

The foodstuffs investigated were: carrots, abbage, dried peas, potatoes, wholemeal read, white bread, lean meat, fat meat and ish. These were given as additions to the asic diet. Finally two composite diets were dministered, the one with fish and white read as the chief ingredients, the other vith fat meat, wholemeal bread and vegeables.

Rectal gas was collected for eight days or each foodstuff for each experimental ndividual. The gas was measured for volme and analysed for content of oxygen, arbon dioxide, hydrogen and methane. The xperimental results show that the basic iet did *not* give the lowest values and that iet has no definite influence on the quanty and composition of rectal gas.

A diet rich in both fat and vegetables may ossibly give some increase in volume, artly due to increased peristalsis, partly due to a real increase in the proportion of fermentation gases.

Isolated, high values for fermentation gases were found without relation to specific foodstuffs or other external factors.

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INTRA-ARTERIAL BLOOD PRESSURE MEASUREMENTS DURING ELECTRO-SHOCK CONVULSIVE TREATMENT WITH AND WITHOUT A MUSCLE RELAXANT (SUCCINYLCHOLIN IODIDE)

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Electro-shock convulsive treatment (ECT) has been, and still is, a frequently used therapeutical method in psychiatric hospitals. The contra-indications are often difficult to estimate, especially as to the cardiovascular system, and accordingly we believe it to be of interest to determine the stress which ECT produces on the cardiovascular system.

Several investigations have been carried out on the circulation during ECT. Silfverskiöld & Åmark (1943) demonstrated that ECT produces a considerable rise in the arterial blood pressure. More recent authors are of the same opinion. Laboucarie & Benazet (1953) postulated that after simple ECT there is an almost constant sudden rise in arterial tension, especially high in true hypertensives.

Deshaies & Renault (1948) were of opinion that the most common blood pressure change following ECT was hypotension.

Brown, Brown & Hines (1953) are of the opinion that the cardiovascular changes following ECT are independent of the convulsions and result from the effect of the electro-shock stimulus alone. They found that curare (tubocurarine) *per se* does not produce marked blood pressure changes in man.

On this point there are differing opinions as to the effect of curarization on the arterial blood pressure in ECT. Woolley (1944) observed blood pressures as high as 180 to 290 mm Hg, despite administration of curare. Donovan (1947), Igersheimer & Stevenson (1951) also reported a marked rise in blood pressure although curare had been given. Massion-Verniory, Dumont, Nicaise & Reinhold (1947) reported that during og after ECT under curare the arterial pressure sometimes increases or decreases. Hobson & Prescott (1947) found a slight rise in blood pressure when curare and thiopental sodium were given prior to the electro-shock.

Holmberg, Thesleff, von Dardel, Hard, Ramquist & Pettersson (1954) have investigated these conditions, using a mechano-electronic transducer manometer. The arterial pressure was found to rise during ECT. When the muscular spasms are eliminated with the administration of succinylcholine and oxygen, a slower and more even rise is noted, the difference between the maximums with these methods is almost significant. It is concluded that with the use of a muscle relaxant and oxygen it is possible to reduce significantly the strain on the circulatory organs caused by ECT.

Several investigations have been carried out on the effect of succinylcholine on blood

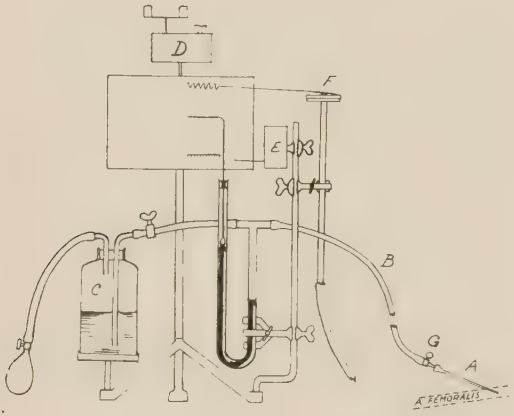

Fig. 1. Ludwigs kymograf. A: Artery needle. B: Plastic tube. C: Pressure-bottle. D: Clockwork. E: Watch marking the time in seconds. F: Membran pelotte registering the respiration.

pressure. Bovet, Bovet-Nitti, Guarion, Longo & Fusco (1951) reported that small or moderate doses of succinylcholine had no effect on the blood pressure and heart rate when artificial respiration was administered. Only large doses (10 mg/kg) caused a transient rise in blood pressure, accompanied by tachycardia. No effect on the ECG was observed. Castillo & de Beer (1950) stated that, in the cat and dog, succinylcholine caused no appreciable changes in blood pressure, except when respiratory arrest with asphyxial hypertension occurred. Ginzel, Klupp & Werner (1951) found that succinylcholine caused only an inappreciable rise in blood pressure. Thesleff (1952) has studied the effect on blood pressure in the cat. He found that succinylcholine in doses up to 1 mg/kg of body weight caused no appreciable change in the blood pressure. Succinylcholine in doses of 2—50 mg/kg caused rise in blood pressure.

METHODS

The femoral artery was punctured in 9 female patients and the intra-arterial blood pressure during ECT registered by means of a Ludwig's kymograph.

The femoral artery is punctured beyond ligamentum inguinale by means of an artery needle constructed in a special way (A). This needle is connected with a mercury manometer by a plastic tube (B) filled with a sterile saline solution, to which is added a little heparine. The manometer is adjusted so that the zero line is at the same level as the heart (the precordial area), and the pressure of the fluid above the mercury in one of the manometer tubes, when the artery is not coupled on, is controlled by means of an inflation bulb and a pressure bottle (C) containing the same fluid. Upon the mercury in the other manometer tube there is a little glas floater, this again moving a feather for writing, which marks the mean blood pressure directly on sooty paper fixed to the drum of the kymograph. The drum is moved round by means of a clockwork (D), and a little watch (E) marks the time at the bottom of the paper. A little higher on the paper, signs are marked by stepping on a rubber tube lying on the floor, thus indicating injections and shocks. At the top respiration is registered by means of a membrane (F), by means of a plastic tube attached to a billow tube round the abdomen of the patient. Convulsions during shock are also registered through this tube. One rotation of the drum takes about 4 minutes, but the speed of rotation may be changed by means of a brake wing.

Each patient was given an ECT using the technique of Cerletti and Bini, first without succinylcholin iodide and several days later with succinylcholin iodide. Succinylcholin iodide was given as "Curacit Nyco" in a dose of 0.2 mg succinylcholin iodide per kg body weight. 20 to 30 Joules were used, one or two phasic circuit. In a third series blood pressure was registered in the same patients after injection of succinylcolin iodide, not followed by ECT.

All the patients are diagnosed as schizophrenics, with delusions, hallucinations or of a dementia praecox type. Age varied from 37 to 64 years,

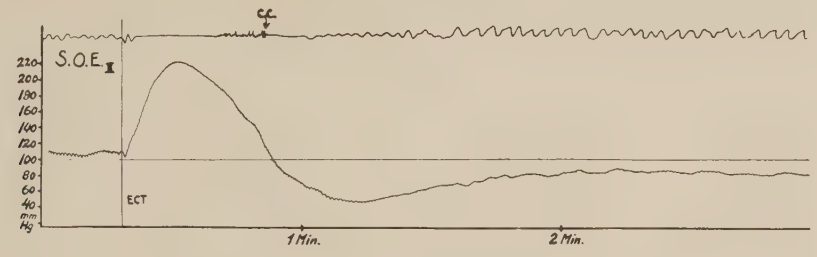

Fig. 2. Mean blood-pressure during electroshock without musle relaxants. ECT: Electroshock. CC: Settlement of convulsions.

average 49 years. Duration of illness varied from 3 to 32 years, average 13 years. All the patients were previously treated with ECT, from one to 92 treatments, average 37.

At the time of investigation none of the patients was receiving drugs, none of them has at any time been treated with chlorpromazine or Rauwolfia drugs. None of them got barbiturates or atropine as premedication. Succinylcholin iodide was injected about 60 seconds before the shock was given. Pure oxygen was administered with intubation and about 40 seconds after injection of succinylcholin iodide, and also after settlement of the convulsions when succinylcholin iodide had been used. In the series where succinylcholin iodide was not injected, oxygen was not administered in any case. In the series where succinylcholin iodide was given alone, oxygen was administered in the same way as if shock had also been given.

RESULTS

Blood pressure during ECT without muscle relaxants

In all the 9 patients we find a uniform curve for blood pressure measurements in the femoral artery during ECT.

Immediately after the shock has been given, the mean blood pressure rises steeply and reaches maximal values after 10 to 20 seconds, then decreasing following a more flattened curve. The decrease starts in the clonic phase of the convulsions, and continues even after the settlement of the convulsions. In 8 of the patients the mean blood pressure falls considerably below the control levels, the curve in these forming a typical "dip" or negative phase. In the last patient the falling part of the curve forms a flat part, before falling rapidly to control level, however, not forming a typical "dip" beyond that level. In the 8 patients mean blood pressures after the "dip" increase slowly towards control level, however, in 5 patients blood pressure after 4 minutes is still beyond control levels. In the other 4 patients blood pressure after 4 minutes is close to control levels. In none of them is blood pressure above control level.

Typical trends in the course of the curve are also the respiratory oscillations in blood pressure (Marey' oscillations) after the settlement of the convulsions and during the rest of the registration (3 to 4 minutes), probably caused by hyperventilation.

One of the patients exhibited a state of anxiety during the awakening, and here we registered an emotional blood pressure rise lasting 45 seconds with a maximal increase of 67 mm Hg(from 108 to 175 mm Hg).

The pulse rate in all the patients demonstrates a marked tachycardia at the top of

Table I.

Case number	1 O.G.	2 B.K.	3 S.M.J.	4 S.O.E.	5 E.P.	6 R.O.	7 M.V.	8 R.S.	9 A.H.	Average
ECT without succ.ch.i.:										
Max. blood pressure	184	190	180	206	186	200	230	196	196	196
Control level	130	104	134	104	112	100	160	128	132	123
Max. blood pressure rise	54	86	46	102	74	100	70	68	64	74
Lowest blood pressure values	98	94	90	54	76	98	108	80	80	87
Greatest difference in blood pressure from max. values to dip.	86	96	90	152	110	102	122	116	116	110
ECT with succ.ch.i.:										
Max. blood pressure	220	196	174	212	160	166	194	200	232	195
Control level	132	126	128	116	108	106	130	112	152	123
Max. blood pressure rise	88	70	46	96	52	60	64	88	80	72
Max. blood pressure after succ.ch.i. before ECT	148	190	140	140	118	128	180	160	210	157
Real rise of blood pressure on ECT	72	6	34	72	42	38	14	40	22	39
Blood pressure rise due to succ.ch.i.	16	64	12	24	10	22	50	48	58	33
Succ.ch.i. alone:										
Max. blood pressure	170	206	162	134	178	156	194	136	166	167
Control level before inj.	130	114	126	110	118	114	164	114	128	124
Blood pressure rise due to succ.ch.i. alone	40	92	36	24	60	42	30	22	38	43

the blood pressure curve, and in 6 we find a relative tachycardia during the whole registration. In the other 3 the pulse rate is found to be normal or slow during the blood pressure fall. It increases, however, after the dip during the awakening period.

From Table I it will be seen that the maximal mean blood pressures during ECT without succinylcholin iodide vary from 180 to 230 mm Hg average 196 mm Hg. The highest mean blood pressure is found in the patient where the control level was highest (essential hypertension). The increase in blood pressures from the control levels varies widely, from 46 to 102 mm Hg, average 74 mm. The highest rises (difference between the maximal blood pressure and control level) are found in patients with normal control levels. From the table it will further be seen that the minimal blood pressure values vary from 54 to 108 mm Hg. If the minimal and the maximal blood pressure values are compared, marked differences are to be found, average 110 mm Hg, the greatest difference being 152 mm Hg. This means that in the course of about 30—40 seconds we get an average fall in blood pressure of 110 mm Hg, *i. e.* a rather considerable fall per unit of time. The average fall in blood pressure beyond control level is 36 mm Hg.

Blood pressure during ECT with muscle relaxants

The curve of the mean blood pressure exhibits a quite different course.

This typical course is seen in 5 patients. After a latent period of 12 to 20 seconds

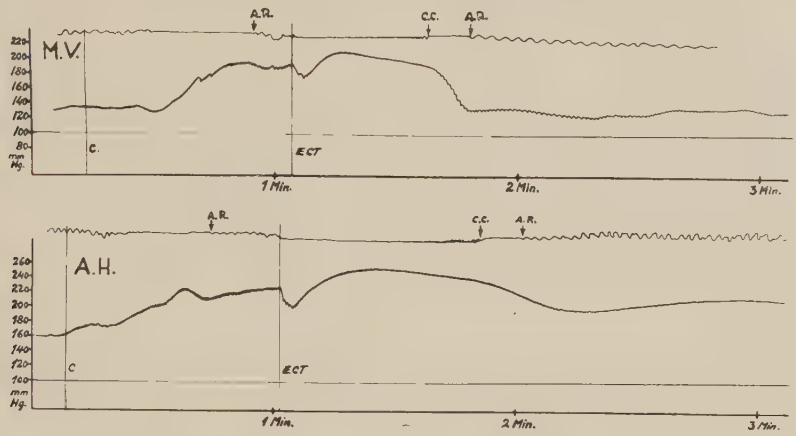

Fig. 3. Mean blood-pressure during electroshock with muscle relaxants. C: Injection of succinylcholin iodide ("Curacit"). ECT: Electroshock. A.R.: Artificial respiration. C.C.: Settlement of convulsions.

from the injection of succinylcholin iodide, and at the same time as apnea commences, mean blood pressure starts to rise, slowly and with some oscillations. 35—45 seconds after the injection oxygen is administered and mean blood pressure continues to rise, reaching no definite maximum when shock is given, about 60 seconds after injection. Immediately after the shock has been given, blood pressure in these 5 patients decreases a little, then increases rapidly untill maximum is reached, staying at maximal values for some seconds, then falling slightly and without a marked dip beyond control level. The duration of the flat part of the curve is dependant on the duration of the convulsions, the fall, however, always starting before artificial respiration is administered.

In all 9 patients the fall is slow and reaches values about control levels. Respiratory oscillations are not seen in these patients. Premature beats after the shock, however, are seen in 6 patients. In one of these, long series of premature beats are seen several minutes after the shock. In one of the patients marked premature contractions are seen after injection of succinylcholin iodide and before the shock, and in another a short period of bradycardia (15 seconds) is seen in the same time interval. On the peak of the curve tachycardia is found in all, in 7 followed by bradycardia in the falling part of the curve. At the moment when shock is given some very slow heart beats are found, corresponding to the earlier mentioned fall in mean blood pressure.

In several control experiments the same pattern of the curve is found in the same patients, the reaction pattern being typical for each patient.

In two cases no muscular reaction followed the ECT ("Blind shock"). The typical curve seen in these cases is demonstrated in Figure 4.

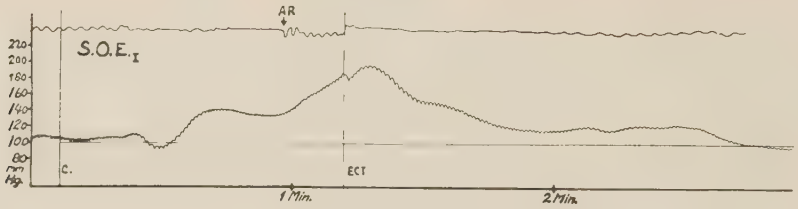

Fig. 4. Mean blood-pressure during electroshock with muscle relaxant and no muscular reaction ("blind shock"). C: Injection of succinylcholin iodide ("Curacit"). ECT: Electroshock. A.R.: Artificial respiration.

The effect of succinylcholin iodide is mainly as in Figure 3, the flattened blood pressure rise, however, and the tachycardia after shock are not seen. On the contrary, blood pressure falls relatively rapid, and bradycardia appears. In one of these patients a new shock was given after 3 minutes, and the continued registration demonstrated a curve corresponding to that in Figure 2. Succinylcholin iodide accordingly had no longer any effect, and the patient got marked convulsions.

From Table I it is seen that the maximal blood pressure values after ECT with succinylcholin iodide vary from 160 to 232 mm Hg, average 195 mm. The highest value here also is seen in the patient where the control level was highest, not in that patient, however, who demonstrated the highest blood pressure after ECT without muscle relaxant. The rise in mean blood pressure from control levels varies from 46 to 96 mm Hg, average 72 mm Hg. The highest rise is also here seen in patients where control levels were normal.

In all patients a blood pressure rise appears before the shock is given, following the injection of succinylcholin iodide. The mean blood pressure at the onset of the shock varies from 118 to 210 mm Hg, the blood pressure rise following injection of succinylcholin iodide varies from 10 to 64 mm Hg, average 33 mm Hg. The real increase following the onset of electro-shock accordingly is markedly less than previously indicated, namely 39 mm Hg on the average.

Blood pressure following succinylcholin iodide

In all 9 patients a marked increase in mean blood pressure is seen; the course of the curve and the maximal rise in blood pressure, however, vary.

In 5 patients there was a similar rise as shown in Figure 5, with a relatively high and rather protracted blood pressure rise, with an oscillating curve falling slowly to control levels. In 4 patients the maximal rise was rather less pronounced and of shorter duration, in two of these, however, the apnea was slight, probably because of a small effect of succinylcholin iodide (slow circulation from the place of injection? rapid destruction?).

The pulse rate was markedly altered from the electro-shock curves. In 7 patients bradycardia is found on the rising part of the curve as long as mean blood pressure shows

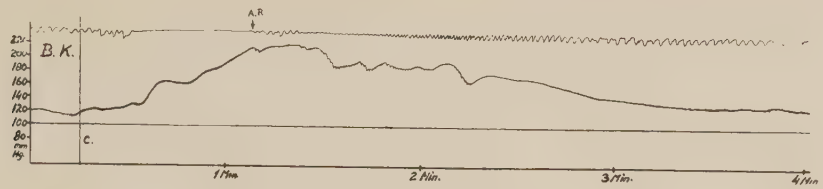

Fig. 5. Mean blood-pressure variations following an injection of muscle relaxant only. C: Injection of succinylcholin iodide ("Curacit"). A.R.: Artificial respiration.

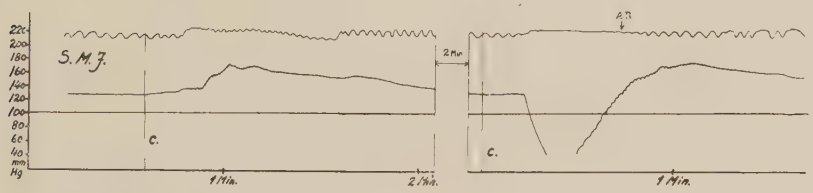

Fig. 6. Mean blood-pressure variations following two repeated injections of muscle relaxant. C: Injection of succinylcholin iodide ("Curacit"). A.R.: Artificial respiration.

high values. Tachycardia, however, is found on the falling curve. Premature beats are aslo commonly found on the peak of the curve (Figure 5). The blood pressure after 4 minutes in 7 patients is about at control levels, in 2 markedly above. Control experiments were carried out, and the same pattern of the curve was seen in the same patient.

In one of the patients where the effect of succinylcholin iodide was bad, repeated injection of the same dose was undertaken 3½ minutes after the first injection.

As will be seen from the curve (Fig. 6), mean blood pressure after 12 seconds fell toward zero, after 16—17 seconds, however, it began to increase, the heart rate being extremely slow. In the interval the patient was pulseless, and without respiration. When the heart beats reappeared, oxygen was administered, and blood pressure increased slowly above control level, then decreasing slightly towards control level.

From Table I it will be seen that the maximal blood pressure varies from 134 to 206 mm Hg, average 167 mm, an average increase of 34 mm. This increase is not remote, and corresponds to the increase in mean blood pressure seen after injection of succinylcholin iodide before shock is given. This latter increase was a bit less, average 33 mm Hg, ascribable to the fact that the maximal effect of succinylcholin iodide does not appear before ECT is given.

DISCUSSION

There seems to be a marked difference in blood pressure patterns when ECT is given without muscle relaxant, and when a muscle relaxant such as succinylcholin iodide is given beforehand. The most essential difference does not seem to be the

maximal blood pressure values, these being about the same in the two series. The blood pressure rise in the first part and the fall in the last part of the shock, however, are more even when muscle relaxants are given, and accordingly the variation per unit of time is much less. Accordingly the heart and circulatory system should be exposed to a less handicapping stress. The blood pressure rise caused by succinylcholin iodide alone when given before the shock, contributes to this accomodation. When muscle relaxants are not given, mean blood pressure after shock sometimes decreases dangerously (Figure 2).

On the other hand it seems justified to conclude that succinylcholin iodide is not an indifferent agent to the circulatory system. Indicative of this are the frequent premature beats found when succinylcholin iodide is given, and also the fact that we in one case with two subsequent injections caused transient heart stop. Blood pressure rise after succinylcholin iodide injection may be explained by the following asphyxia and the $C0_2$-retention, and perhaps by anxiety states, seen in some cases.

When blood pressure values after shock given without muscle relaxants decrease so low and remain beyond control levels, this may be explained by the theory that the circulatory system has had no time to become adapted to the stress situation that the shock represents, while succinylcholin iodide has prepared the organism for this stress. It may be suggested that blood pressure stabilizing mechanisms are mobilized when succinylcholin iodide is used, perhaps *via* the adrenals.

There seeems to be a connection between the duration of the convulsions and the duration of maximal blood pressure, and this seems most marked when succinylcholin iodide is used, even if the fall also here starts before the convulsions settle. This may be caused by the asphyxia, which is more marked when succinylcholin iodide is given.

However, from the blood pressure patterns in "blind shock", it may be seen that the blood pressure decreases rapidly after shock when muscular reaction fails, in spite of continued asphyxia. This indicates that the musclar reaction plays an essential role for blood pressure rise during shock. The marked bradycardia after "blind shock" is also to be noted, perhaps this may be explained by a vagal effect caused by the shock.

In both series there is a marked tachycardia during the shock, probably representing a reaction to stress on the heart *via* outflow of adrenaline, whether muscle relaxants are used or not. This stress seems to be most pronounced where blood pressure is already high.

SUMMARY

By means of a Ludwigs kymograph, intra-arterial blood pressure is registered in the femoral artery during ECT with and without a muscle relaxant (succinylcholin iodide).

It is found that the intra-arterial blood pressure in all patients increases during shock, and that the maximal values for blood pressure are almost the same whether succinylcholin iodide is given or not. The rise in blood pressure, however, is much

slower when succinylcholin iodide is given, and also the fall is much slower and never reaches values beyond control level. When muscle relaxants are not given, there is always a fall to values beyond control level after settlement of the convulsions. The real difference between blood pressure maximum and minimum are accordingly markedly greater when muscle relaxants are not given, and changes in blood pressure per unit of time are also considerably greater. Accordingly it seems that muscle relaxants reduce the stress on the circulatory system caused by ECT.

However, succinylcholin iodide can hardly be an indifferent agent to the circulatory system, as blood pressure rise after injection is seen constantly and premature beats frequently. In one patient a transient heart stop is even seen after repeated injection of succinylcholin iodide.

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VENTILATORY RESPONSE TO MUSCULAR EXERCISE IN PATIENTS WITH CARDIAC DYSPNEA

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(Received for publication December 7, 1955)

Dyspnea is an important symptom in patients with cardiac disease. The classification of these patients suggested by the New York Heart Association (1953) is commonly accepted; it is based on the severity of the subjective feeling of fatique, palpitation, dyspnea or anginal pain caused by ordinary physical activity.

Several authors (Peabody 1922, Nylin 1933, Campbell 1934, Nielsen 1937) have investigated pulmonary ventilation of patients with cardiac disease.

McMichael (1939) has shown that there is a good correlation between pulmonary ventilation and the minute volume of the heart in patients with advanced cardiac disease.

Erikson (1953) has demonstrated that patients with cardiac disease have a far larger increase in pulmonary ventilation on short, moderate exercise than patients with pulmonary disease.

In order to determine the correlation between the patient's subjective feeling of dyspnea and his ventilatory response to a short standardized exercise test, the following investigations were conducted.

MATERIAL

23 individuals without any symptoms of cardiopulmonary disease and 20 patients suffering from heart disease were studied. The diagnoses of the heart patients were as follows:

Rheum. valv. heart disease	8
Hypertensive heart disease	4
Congenital heart disease	4
Paroxystic auric. fibrillation	3
Auric. flutter	1
	20

The heart patients were classified according to the classification of the New York Heart Association (1953).

METHODS

During the experiments the subject was seated in a comfortable chair and inspired room air. The expired air was collected partly in plastic bags and partly in a Tissot spirometer. A bicycle ergometer was placed in front of the chair (Fig. 1). A noseclip prevented the subject from breathing through the nose.

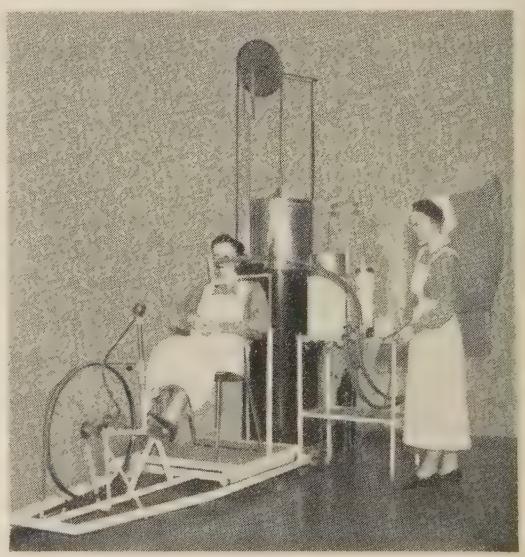


Fig. 1. A subject during the exercise test.

The expired air was collected in a plastic bag during 10 minutes of rest; then the subject did external work on the bicycle ergometer of approximately 500 kg-m in 1 minute. The air expired during this exercise and the following 9 minutes was collected in the Tissot spirometer, and the volume read each minute. The expiratory tube was then switched to a second plastic bag and the expired air collected in this bag for a period of 10 minutes. The resistance to breathing in this system was only 2 to 3 mm water.

After the experiment the volumes of air in the plastic bags were determined by emptying into the Tissot spirometer.

Duplicate exercise tests were done on separate days.

In order to exclude patients with acidosis or alkalosis the alkali reserve was determined immediately before each experiment. It was also determined at the end of each experiment since excessive hyper- and hypoventilation will result in a corresponding change in alkalireserve.

RESULTS

The ventilatory response to the exercise test of 23 subjects without symptoms of cardiopulmonary disease:

The resting ventilation was found to vary from 5.8 to 13.0 l/min. The increase in ventilation during the exercise and the recovery phase was between 15 and 20 liters in 11 subjects; the lowest and highest values were 6.5 and 27 liters respectively (Table I).

Table I. *Increase in ventilation during the exercise (500 kgm/min.) and recovery phase of 23 subjects without any symptoms of cardiopulmonary disease.*

	Increase in ventilation in liters				
	5 to 10	11 to 15	16 to 20	21 to 25	26 to 30
Number of subjects:	1	7	8	6	1

The difference in ventilatory increase in repeated examinations on the same subject averaged 3.1 liters; the largest difference was 6.5 liters.

Fig. 2 shows the results of two experiments on a patient without symptoms of cardiopulmonary disease.

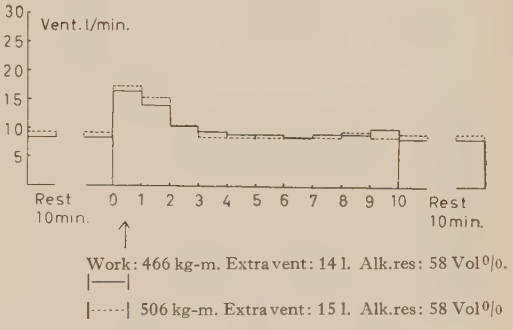


Fig. 2. Pulmonary ventilation during exercise test. (1 minute's work on bicycle ergometer.) Patient No. 13 ♂ Age: 19. Ulc. duod. Dyspnea 0.

The ventilatory response to the exercise test of 20 patients with various degrees of cardiac disease (see material):

The resting ventilation varied from 5.1 to 12.3 l/min. The increase during the exercise and the recovery phase varied from 21.5 to 65 liters (Table II).

The difference in ventilatory increase on repeated examinations of the same subject averaged 3.7 liters; the greatest difference was 9.5 liters.

Fig. 3 shows the reults of two experiments on a 55 year old female patient with congenital heart disease.

DISCUSSION

The preliminary results of this investigation show that the pulmonary ventilation during rest is approximately equal in normal

Table II. *Increase in ventilation during the exercise (500 kg-m/l min.) and recovery phase of 20 patients with varying regrees of cardiac disease classified according to criteria set by the New York Heart Association, 1953.*

		Increase in ventilation in liters								
		21 to 25	26 to 30	31 to 35	36 to 40	41 to 45	46 to 50	51 to 55	56 to 60	61 to 65
Number of patients	Class I.	3	4	1						
	» II.			1	2	3	1	1		
	» III.							3		1

individuals and in patients with cardiac disease, classified in dyspnea groups I to III.

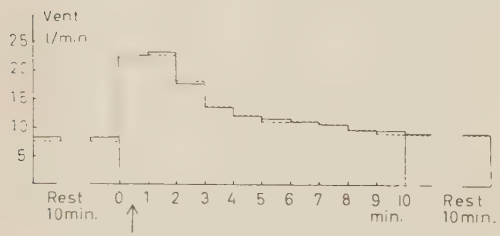
There is a large difference between the ventilatory responds to exercise of normal subjects and patients with cardiac disease (Table I, II and Fig. 4).

When performing the same exercise the cardiac patients show a far greater increase in pulmonary ventilation than the control individuals.

With progressing invalidization by cardiac disease, as classified by the New York Heart Association, increasing values for pulmonary ventilation during and after exercise are found. This indicates that the dyspnea of cardiac patients during exercise is due to excessive increase of the pulmonary ventilation.

Further investigations will show to what extent the subjective feeling of dyspnea of cardiac patients performing a short standardized exercise is correlated with increased pulmonary ventilation.

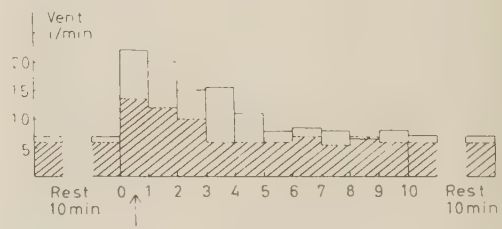
If this correlation is verified, the classification of cardiac disease will be facilitated and the evaluation of the effect of treatment improved.



Work: 472 kg-m. Extra vent: 51,5 l. Alk.res.
before: 53 vol%, after: 48 vol%.

472 kg-m. Extra vent: 54,5 l. Alk.res.
before: 54 vol%, after: 56 vol%.

Fig. 3. Pulmonary ventilation during exercise test. (1 minute's work on bicycle ergometer.) Patient No. 26. ♀. Age 55. Vit. congenit. cord. Dyspnea III.



No. 51. Work: 500 kg-m. Extra vent: 49,5 l. Alk.res.
before: 54 vol%, after: 50 vol%.

No. 15. 455 kg-m. Extra vent: 17,5 l. Alk.res.
after: 59 vol%.

Fig. 4. Pulmonary ventilation during exercise test. (1 minute's work on bicycle ergometer.) Patient No. 510 ♀. Age: 40. Vit. valv. cord. rheum. Dyspnea II. Patient No. 15 ♀. Age: 50. Ulc. duod. Dyspnea 0.

SUMMARY

The pulmonary ventilation of cardiac patients and patients without symptoms of cardiopulmonary disease has been determined during rest, a short standardized exercise (500 kg-m performed in 1 minute) and in the recovery phase.

The increase in ventilation caused by this exercise was found to be greater in the cardiac patients than in the control subjects. On repeated examinations this ventilatory increase was found to be rather constant, and well correlated with the degree of dyspnea.

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URIC ACID IN HUMAN BLOOD CORPUSCLES AND PLASMA

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The distribution of a substance between blood corpuscles and plasma is most often calculated on the basis of haematocrit determination and quantitative analysis performed on whole blood and plasma. Such examinations have all shown that in human blood uric acid is present in both corpuscles and plasma; nevertheless, there is no general agreement on the ratio between the concentrations. Thus Folin & Svedberg (1930), Heller (1935) and Benedict (1931) found the uric acid concentration — by volume — in the blood corpuscles to be 22 per cent, 54 per cent and 72 per cent, respectively, of the concentration in plasma.

The analysis of *whole blood* for uric acid is complicated by two principal sources of error: Deproteinisation causes a considerable and varying loss of uric acid, and non-enzymatic reduction methods give too high analytical results. By analysing *plasma,* loss through deproteinisation can be avoided (Jørgensen & Poulsen, 1955), but then certain colorimetric uric acid determinations will give too high values and others too low (Lous & Sylvest, 1954). Such methods are, therefore, in principle, unfit for investigations of the distribution of uric acid between the blood corpuscles and plasma.

Using the enzymatic-optic method, a specific determination of uric acid in non-deproteinised blood fractions is obtained through the extinction decrease occurring at 292.5 mμ (maximum absorption of uric acid) after addition of uricase. Plasma dilutions in a suitable buffer solution can be submitted to direct analysis. The same is true of hemolysed whole blood, provided the uric acid concentration is so high that the hemolysate can be adequately diluted. Often, however, the concentration in human blood is so low that the intensity of the ordinary hydrogen discharge lamp used in the Beckman spectrophotometer is not sufficient for working with a suitable small slit-width. In principle the problem must be solved by using a lamp of high intensity within the range of about 290 mμ.

The object of the present study was to devise a method allowing us to use the ordinary Beckman equipment. In this the blood corpuscles are washed with saline, thereby transferring the uric acid to a medium in which it can be determined without deproteinisation.

ANALYTICAL METHOD

The experiments were made on freshly drawn, heparinised, human venous blood. The uric acid determinations were done by the enzymatic-optic method devised by Prætorius (1949) and modified by Prætorius & Poulsen (1953).

Washing of uric acid from blood corpuscles

Two conditions are necessary for uric acid to be washed out of the blood corpuscles. Firstly, that these are permeable to uric acid under the given experimental circumstances, and secondly, that diffusion equilibrium between corpuscles and wash fluid is obtained within a few hours, so that loss of uric acid, *e.g.* by bacterial processes, can be ignored. The following experiment showed these requirements to be fulfilled: 30 ml of heparinised venous blood was centrifuged (1000 times gravity) for 10 minutes, and as much plasma as possible was siphoned off. In a graduated cylinder 1 per cent sodium chloride solution was added to the blood corpuscles plus remaining plasma, to a volume of 110 ml. The cylinder was placed in a mechanical rocking apparatus. Samples of 10.0 ml were withdrawn at suitable intervals and centrifuged. 2.0 ml of the supernatant were pipetted off for analysis, while the blood corpuscles and the remaining supernatant fluid were returned to the main portion for further rocking. Table I shows that the concentration of uric acid in the fluid increased only the first three hours. Continued rocking for 24 hours left the extracellular uric acid concentration at a constant level.

Table I. *Concentration of uric acid in the extracellular aqueous phase after shaking of the blood corpuscles from 30 ml of whole blood with 1 per cent sodium chloride solution. Total volume 110 ml. Temperature 25° C.*

Hours	¼	½	¾	1	1½	2	3	5½
µmol/l ..	16.1	18.7	21.1	23.0	25.5	25.7	27.3	27.0

Similar results were achieved by repeating the experiment.

Calculation of the uric acid content of blood corpuscles

If we take the constant concentration of uric acid in the wash fluid to indicate equilibrium between the intracellular and the extracellular aqueous phase, the concentration of uric acid in the blood corpuscles *prior to* the washing must have been:

Concentration of uric acid in the wash fluid plus that in the washed corpuscles minus that in the non-removed plasma:

$$\left(V-\frac{V_b \cdot H}{100}\right)\cdot C_w + \frac{V_b \cdot H}{100}\cdot C_c - \left(\frac{V_b \cdot (100-H)}{100}\right)\cdot C_p$$

µmol uric acid/30 ml of blood (I)

where:

V = total volume (liters) of the suspension
V_b = volume (liters) of the blood sample used
V_p = volume (liters) of the removed plasma
C_w = concentration (µmol/liter) of uric acid in wash fluid
C_p = concentration (µmol/liter) of uric acid in plasma
C_c = concentration (µmol/liter) of uric acid in washed corpuscles
H = hematocrit value (%)

All these values are known, except Cc, which can be estimated from the following equation:

$$C_c = \frac{C_w \cdot 0.8 \cdot 72}{100} \qquad \text{(II)}$$

assuming that the corpuscles contain 72 per cent water, and that the uric acid, like certain other anions, is distributed between the intracellular and the extracellular aqueous phase according to Donnan's law at a ratio of 0.8. Thus the uric acid content of the blood corpuscles prior to the washing can be approximated by inserting (II) in (I).

Since the error of the uric acid measurements amounts to a few per cent at most, any appreciable error entering into (I) can be attributed chiefly to errors of Cc and H. The error of H is due to the fact that the cell volume is determined on freshly withdrawn blood and is unlikely to remain constant during the washing. The relatively great error of the second and third terms as well as of $\frac{V_b \cdot H}{100}$ in (I) can be neglected by removing as much plasma as possible and by using a large volume of wash fluid in proportion to the

Table II. *Uric acid in plasma and blood corpuscles from 10 subjects.*

Subject	Uric acid conc. in μmol/l		$\frac{C_{\text{uric acid (cells)}}}{C_{\text{uric acid (plasma)}}}$	Hematocrite in per cent	Uric acid conc. in whole blood in μmol/l (calculated)
	Plasma	Cells			
P.R.B.	337	186	0.552	42.5	273
S.J.	308	143	0.464	42.0	239
K.L.	276	148	0.536	44.5	219
H.H.L.	280	165	0.589	41.0	233
P.M.	275	206	0.750	45.0	244
A.T.N.	259	149	0.575	41.0	214
P.P.	291	147	0.505	45.0	226
An.S.	168	92	0.548	35.5	141
Ar.S.	331	177	0.535	43.0	265
K.S.	198	99	0.500	46.5	152
Average	272	151	0.555	42.6	221

volume of corpuscles. On the other hand, the volume of the wash fluid must not be larger than that the uric acid determination gives suitably high values for $-\Delta E_{292.5\ m\mu}$. In all our experiments we used corpuscles from 30 ml of blood and added 1 per cent sodiumchloride solution to a total volume of 110 ml.

RESULTS

Blood samples from 10 adult subjects were examined by the method here described. The results are recorded in Table II.

DISCUSSION

The experiments demonstrate that human blood corpuscles contain considerable amounts of uric acid. The average distribution ratio — by volume — between cells and plasma (0.55) agrees with that found by Heller (0.54). Individual variations occur which prevent any calculation of the uric acid concentration in whole blood from that in plasma. These variations might be expected by assuming that uric acid is present as an anion distributed according to Donnan's equation:

$$\frac{[\text{uric acid}] \text{ in cell water}}{[\text{uric acid}] \text{ in plasma water}} = k.$$

Since k is a constant (usually 0.7—0.8) depending, among other things, on the pH and the degree of oxidation of the hemoglobin, the distribution ratio will vary among and within individuals. Furthermore, the distribution ratios, as calculated in our series of experiments (Table II) are not true distribution ratios in the strict physico-chemical sense, but merely the ratios — *by volume* — between concentrations in cells and plasma. Consequently, variations in water content in the cells will further explain the distribution variations demonstrated in our experimental subjects. The water contents of the plasma and blood corpuscles were not determined in this study, thus we must confine ourselves to the gross, approximate calculation that can be carried out with the mean values at the

ottom of Table II. According to Albritton 1952) the water contents of blood corpuscles and plasma may be set at 72 70—75) and 94 (93—95) per cent (w/v), espectively. By using these figures on our xperimental results we get:

Concentration of uric acid in blood corpuscles (C_{cells})
$= 151/0.72 = 210$ μmol/l H_2O

Concentration of uric acid in plasma (C_{plasma})
$= 272/0.94 = 280$ μmol/l H_2O

nd from this:

$$C_{cells}/C_{plasma} = 0.75$$

albot & Sherman (1936), using a chemical ethod, found the ratio between the uric cid concentrations in the aqueous phases f cells and plasma to average 0.60.

Regarding the state of uric acid in human lood, we know only that the *plasma* uric cid is present as the free ion. This is evient from the results of unpublished investigations by Prætorius and by Poulsen, ho have shown that the specific uric acid pectrum of deproteinised plasma disappears completely after addition of uricase. ittle is known of the state of the uric acid blood corpuscles. Newton, Davies & enedict (1922) isolated uric acid riboside rom beef blood corpuscles. They believed is substance to be present in human blood, o, though probably in smaller amounts. was on this basis that Kalckar (1947) resumed that human blood corpuscles do ot contain free uric acid at all. Considerg these aspects, it may be doubted whether e "free" uric acid that we found in the ash fluid from blood corpuscles had originally been present in the free state. The possibility exists that the uric acid in the corpuscles is present entirely or in part as riboside or ribotide, which split off uric acid enzymatically during the washing according to the experimental procedure. Experiments are currently under way aiming at elucidating this problem.

SUMMARY

Using an enzymatic-optic method, we have shown that human blood corpuscles yield uric acid when washed with 1 per cent sodium chloride solution, reaching equilibrium in approximately 3 hours. The original content of uric acid in the blood corpuscles has been calculated from the concentration at equilibrium. In 10 subjects the ratio of uric acid per liter of corpuscles to uric acid per liter of plasma was found to average 0.55 (ranging from 0.46 to 0.75).

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PAPER ELECTROPHORESIS OF PORPHYRINS

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Although several methods of paper chromatography of porphyrins and their esters have been published (Nicolas & Rimington, 1949, 1951; Chu, Green & Chu, 1951; Eriksen, 1953; Falk & Benson, 1953; Chu & Chu, 1954; Kehl & Günther, 1954) no separation of porphyrins or their esters based on paper electrophoresis has been proposed. The only available paper dealing with electrophoresis of porphyrins is the brief communication of Papatasmatis & Kench (1952) who performed iontophoresis of porphyrins in M/15 buffer of pH 8.0 at 200 v employing an agar gel as supporting medium and using water cooling. Their method was rather laborious because meticulous purification of the agar was necessary, but satisfactory separation of porphyrins with different numbers of carboxyl groups was obtained, whereas no sepearation of either coproporphyrins or uroporphyrins I and III could be achieved in this way. The speed of migration increased with the number of carboxyl groups, *i. e.*, uroporphyrin moved ahead and protoporphyrin at the tail of the sequence of porphyrins — the opposite of what is found in paper chromatography with the collidine-water system.

As paper electrophoresis has been developed into a simple technique of considerable efficiency in the case of serum proteins, there was good reason to believe that the method could also be applied to porphyrins, and this was found to be the case. Actually, the method is more simple for porphyrins than for proteins because the latter require staining to make the various bands visible while the porphyrins can be mabe visible simply by irradiation with Wood's light.

The method requires porphyrin solutions of a concentration of about 5 μg per ml or more, so that only concentrated urines from cases of porphyria can be subjected to electrophoresis without previous concentration. An easy means of obtaining concentration of less concentrated solutions is adsorption to fullers' earth at pH 3—4 with subsequent eluation with ammonia.

MATERIAL

In our experiments we used urine from a steer and some pigs with congenital porphyria (cf. Jørgensen & With, 1955) as well as human urine from various patients including one with acute porphyria.

METHODS

Wood's light

Osram analytical bulb, type HQV 300, 75 w.

Electrophoresis apparatus

Glass tank 30 × 20 × 10 cm with glass lid and two perforations to let two wires pass. The bottom of the tank was filled with water to ca. 1 cm's height to secure sufficient saturation of the air. Placed at the bottom of the tank along its long sides were electrode glass vessels ca. 25 cm long, ca. 5 cm broad, and ca. 3 cm high. Platinum wire, supported by rods of glass or plastic coated wire, was used as electrodes. The rods were formed to fit into the electrode vessels. The electrode wires were then connected to the wires from the transformer passing through the glass tank. The source of power was a transformer of the usual type used in paper electrophoresis of proteins, equipped with ampère- and voltmeter. The paper was supported by two glass rods running parallel to the long side of the tank from one of its short sides to the other and placed ca. 3 cm below the lid of the tank. The rods were supported by suckers of rubber or plastic which could be moved at random.

Buffer

M/20 barbiturate buffer pH 8.6 or M/15 phosphate buffer (Sørensen) of pH between 7.4 and 7.6. 200—250 ml were placed in each of the electrode vessels.

Paper

Whatman I was found to be the most suitable. As a rule sheets 20—25 cm × 25 cm were employed; but if only a few porphyrin samples are to be analysed, strips 5 cm wide or even less may be used.

Voltage

7.5—8.0 v per cm were found most suitable, corresponding to ca. 200 v for the dimensions of paper employed, but up to 500 v could be applied with correspondingly increased speed of movement.

Current

5—10 mA for the barbiturate buffer, 10—20 mA for the phosphate buffer. To maintain constant voltage the current sometimes had to be increased during the course of the electrophoresis. The separation of the porphyrins was, however, not disturbed by minor variations of voltage or current

Time

1 to 3 hrs. will usually give a satisfactory separation, but it is recommended to study the paper i Wood's light during the run in the tank; in this way one can follow migration of the porphyrin spots during the procedure. This requires a transportable Wood's lamp, which can be moved around the tank; but such an arrangement is easily built up.

Application to the paper

The best method is to apply the porphyrins to the dry paper. A line is drawn with pencil ca. 5 cm from one of the 20—25 cm long sides of the paper sheet. On this line the porphyrin solutions or urines are applied, *e. g.*, with Pasteur pipettes. The diameter of the spots should be ca. 5 mm, and the free space between them ca. 1 cm or more. The spots are dried in a current of hot air. The fluorescence of the dry spots is controlled in Wood's light, and if it is not sufficient a new spot of solution is placed exactly on the top of the first. Then this is dried and controlled, and in this way one may continue until the fluorescence is sufficiently strong. In the case of untreated urine spots with red fluorescence may be impossible to obtain. Here 3—4 applications on top of each other are performed. Now the contours of the spots are drawn with pencil and each spot marked with pencil for identification. Then the markers are applied (cf. below) and after this the paper is wetted with buffer by dipping, but during this the zone of the porphyrin spots and 2 cm on each side of the pencil line should be left dry to prevent flowing. The surplus moisture is removed by blotting with filter paper, and the dry porphyrin zone is wetted by pressing against a piece of filter paper wetted by dipping into the buffer, after the surplus moisture has been removed. By gentle pressing of the dry porphyrin zone with two clean, moistened strips on filter paper after each other sufficient wetting to run the electrophoresis has been obtained, and the paper can be placed in the tank

Markers

Porphyrin markers must be placed on each sheet f paper to control the efficiency of the run. We ound that the type of markers used by Nicolas & imington (1949, 1951) to control their lutidine-ater paper chromatography was suitable for elec-ophoresis too. These markers consist of solutions f purified uro-, copro-, and protoporphyrin in 2 N mmonia; a concentration of 25—50 μg per ml suitable. If stored in the dark at room tempera-ure they are stable for many months. On each heet of paper a spot of uro-, one of copro-, and ne of protoporphyrin are applied alongside of ach-other. The fluorescence of the marker spots controlled in Wood's light.

Placing the paper in the tank

The moist paper with the porphyrin spots along e pencilled line is cautiously removed from the lter paper; in the moist condition the paper is ather fragile. It is placed in the tank with great aution with the end with the porhpyrin spots owards the cathode and the other end towards the node. The glass rods support the paper. By oving the rods one makes certain that the tension f the paper is appropriate, that the two ends are laced in the middle of the electrode vessels, and hat they dip ½—1 cm below the surface of the uffer solution without touching the sides of the lass vessels. Now the lid is placed on the tank. losure sufficient to insure water saturated air is btained with silicone grease. Then the current turned on. After the electrophoresis the paper trips are dried on a radiator and the porhpyrin pots marked with a pencil in Wood's light.

Preparing concentrates with fullers' earth

The urine — or solution — is acidified (control ith congo paper) by means of conc. HCl or acetic id. Then fuller's earth in an amount of ca. 5 g per 100 ml is added, followed by thorough ixing. We employed Merck's Bleicherde (Deut-he Ware,. 1901). The earth is of brownish color nd violet fluorescence in wet condition, but even nall quantities of porhpyrin adsorbed to it give plainly visible purple fluorescence. Most of the porphyrin is removed from the solution by the first adsorption — a fact readily controlled by carrying out a second one and studying the adsorbates in Wood's light. The fluid is sucked off on a Büchner funnel and the adsorbate washed with distilled water on the funnel and sucked dry. Then the porphyrin is eluated by suspending the earth in as little 2 N ammonia or 10 per cent aqueous pyridine as possible, agitating for a few minutes and centrifuging. The filtrate shows a deep red fluorescence in the case of porphyrin-rich material, and practically no fluorescence is left on the fuller's earth.

RESULTS

A very clear separation of porphyrins with 4 or more carboxyl groups is achieved with this technique while the porphyrins with less than 4 carboxyl groups are only separated to a limited degree. This may be improved by employing longer periods of electrophoresis, but according to our experience even 10 hrs. or more do not give a satisfactory separation of proto- and coproporphyrin. We have tried buffer solutions of pH 6.5 to 8.6 and the following different papers: Whatman I, Whatman 25, Whatman 7312—3, Munktell 20, Sleicher & Schüll No. 595, but this did not improve the result; the best separation and the most well-defined spots were found with Whatman I paper and m/20 barbiturate buffer of pH 8.6.

The porhpyrins are dissociated as acids as the pH in question, being on the alkaline side of their isoelectric points. Consequently they occur as anions and run towards the anode; therefore the end of the paper sheet with the porphyrin spots should be placed at the cathode.

Under our experimental conditions we found that uroporhpyrin ran 10—15 cm, coproporphyrin 0.5—2 cm, and protoporphyrin 0.2—0.3 cm. This is the case if the

samples are applied from ammonia, pyridine, neutral or sligthly acid solution. If they are applied from strongly acid solution the porphyrins are delayed in their run because it takes some time before the buffer solution neutralizes the acid, and before this takes place no migration towards the anode is seen because no porphyrin anions are present.

We have compaired electrophoresis runs with lutidine runs from the same solutions and always found the same spots with the exception that the electrophoresis could not separate coproporphyrin I and III contrary to what is the case with lutidine chromotography with 2,6 lutidine; electrophoresis gives a more pronounced separation of the porphyrins with high carboxyl numbers while the lutidine chromatography gives a more marked separation of the porphyrins with low carboxyl numbers. Thus the two methods supplement each other. The electrophoresis has the advantage that it requires considerably less time, 1—3 hrs. as against 16—18 hrs. (with descending lutidine technique).

We have tried to develop the electrophoresis to a quantitative micro-method of measuring uroporphyrin in a mixture of porphyrins. But, unfortunately, this did not work because of considerable losses in the course of the adsorption and eluation from the paper. The porphyrins are readily eluated from the dried paper with dilute HCl or ammonia, but the recovery percentage was found to be 50 or below in experiments with solutions of pure uroporphyrin.

SUMMARY

A simple method of micro-separation of porphyrins by means of paper electrophoresis is described. Whatman I paper, m/20 barbiturate buffer of pH 8.6, 200 v (7.5—8.0 v per cm), 5—10 mA ,and 1—3 hrs. are recommended. The porphyrins run as anions and are applied to the moistened and partially dried paper from alkaline, neutral or sligthly acid solutions. The run may be directly controlled by irradiation of the tank with Wood's light. Under these conditions uroporphyrin runs 10—15 cm, coproporphyrin 0.5—2 cm, and protoporphyrin 0.2—0.3 cm. Each run must be controlled by application of porphyrin markers.

ADDITION TO THE PROOF

Since this paper was presented for publication Toa Heikel has published a similar method in this journal (vol. 7, p. 347). This writer found a recovery of 80—90 per cent of porphyrins applied to paper. I have since tried to reproduce her recovery experiments, employing exactly the conditions she describes, with the difference that I used purified porphyrin extracts controlled by electrophoresis and lutidine chromatopraphy instead of solutions of pure, crystalline porphyrins. Further I read the porphyrins spectrophotometrically (cf. my note in this journal vol. 7, p. 194) and not fluorimetrically. My experiments still showed, unfortunately, a very low recovery — below 50 per cent as in the experiments described in my paper. This discrepancy may be due to Heikel's use of pure porphyrins; but as the method is to be applied to rather impure solutions, my experiments seem to me the more appropriate. Perhaps also the fallacy of fluorimetric measurements can explain some of the discrepancy.

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METHOD FOR CLINICAL STUDY OF INTRAVASCULAR ERYTHROCYTE AGGREGATION BY MICROPHOTOGRAPHY OF THE SMALL VESSELS OF THE CONJUNCTIVA BULBI

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In recent years increasing attention has been focussed on the intravascular aggregation of erythrocytes. That the erythrocytes tend to aggregate in practically all diseases has long been known. However, it was not until 1921, when Fåhraeus published his paper on the stability of the suspension of blood that the significance of such aggregation in desease was realised. In health the blood cells repel one another and therefore occur as separate elements in the blood stream; in disease, however, they tend to form clumps of varying size and firmness. Fåhraeus showed that this clumping is due to an increase in the percentage of larger protein molecules. Such aggregation can be produced experimentally by intravenous injection of thrombin, fibrinogen, gelatin or high molecular dextran fractions, for example (Thorsén & Hint, 1950).

According to Fåhraeus, intravascular aggregation increases the relative rate of flow of the erythrocytes in the large paracapillaries but a simultaneous increase occurs in the resistance offered by the small capillaries and with formation of embolus-like aggregates of red cells. These aggregates become lodged in the vessels, especially when the blood pressure is low. The total interface between the erythrocytes and the plasma is decreased. This in turn reduces the reaction surface of certain enzymatic processes and decreases the O_2 and CO_2 carrying power of the blood cells. The fundamental investigations of Knisely, who has been working on this and related problems since 1941, have contributed considerably to our knowledge in this field. In addition to the aforementioned deleterious effects of erythrocyte aggregation he showed that the increased resistance offered to the passage of the clumped erythrocytes through the capillaries must decrease the vitality of the tissue by anoxia, especially if small vessels become obstructed. Knisely also showed that the erythrocyte aggregates are gradually destroyed by phagocytosis in the liver and spleen with consequent decrease in the number of red cells and possibly a certain degree of hypoproteinemia. Knisely believes that sedimentation and deposition of large erythrocyte clumps in veins with decreased rate of circulation might be a possible cause of thrombosis (Warner, 1948).

As trauma is followed by erythrocyte aggregation, it is perhaps but natural that serious attempts have been made to find effective prophylactic and therapeutic

gents. Intravascular clumping can be produced experimentally by the injection of igh molecular dextran fractions. Such lumps can afterwards be broken down by he injection of low molecular fractions.

Investigation and objective analysis of he findings made in this field, which might omprise important problems bearing on hrombosis, the development of post-traumatic conditions, post-operative anuria, the iepatorenal syndrome and the reaction of he blood to hypothermia, requires an atraumatic charting of the development of the lumping *in vivo* in animals and in human eings not subjected to trauma. The method escribed by Knisely consists of direct obervation of the vessels in the conjunctiva ulbi and estimation of the size and passage f the clumps. Such a method is far too ubjective for registration purposes. Therefore serious attempts have been made to levise objective methods capable of complementing the valuable direct observation f the condition of the blood stream.

Continuous registration and comparison an be secured by means of photography, but he microphotographic equipments hitherto vailable do not fulfil the requirements of uch a method.

A satisfactory photographic method should, f possible, fill the following requirements:

1. An optical system permitting rapid eries of momentary exposures of a moving bject in sufficient magnification.

2. A source of light permitting fine justment and short exposure time without inconvenience or injury to the patient.

3. It should be easy to handle and permit mbulant and bedside photography.

EARLIER STUDIES

Knisely (1945), who introduced the method of studying the vessels in the bulbar conjunctiva with the aid of a stereomicroscope, reported that he did not succeed in producing photographs of sufficient sharpness and magnification. He published a number of drawings.

Louise Warner (1947) published a film on intravascular settling of sludged blood in the large venules. This film was, however, taken with transillumination of the mesentery of the animals. The results of any methods requiring traumatisation, such as surgical exposure of an intestinal loop, cannot be accepted as applicable to normal conditions.

In his very extensive survey of sludged blood Bigelow (1949) referred to various recording methods. He gave reproductions of pictures taken with transillumination and with a total magnification of up to 220 ×. The films were taken with an exposure time of 20 millieseconds and even in capillaries with a diameter of 10 μ the picture is blurred by movement of the cells.

Zilliacus (1952) photographed the vessels of the bulbar conjunctiva through an ordinary microscope tube provided with an ultrapak objective and ring condenser. The apparatus is focussed with oblique illumination, but the film is taken with a flash bulb via the ring condenser. No optical data are given.

Bloch & Petry (1953) described an apparatus for taking moving pictures of the blood flow through the conjunctival vessels. It is apparent from their article that their studies were carried out on vessels with a diameter of more than 50 μ. We have reproduced the experiments as closely as possible and found that their apparatus could not be used for investigation of the capillaries.

Ditzel (1954) devised a method for photographic reproduction of the smaller conjunctival vessels. His investigation deals mainly with the appearance of the vessels in diabetics, for which reason he contented himself with a fivefold magnification on the negative. This is not sufficient for the study of single clumps of erythrocytes. As a source of light he used a flash bulb in an ordinary reflector. The pilot light consisted of an ophthalmologic lamp placed as near the

flash bulb as possible. The value of this apparatus was limited by uncontrolled reflexes. This disadvantage was overcome by an illumination system designed by Winkelman & Warmoltz (1954), who used a spiral electron flash bulb with a central hole 8 mm in diameter. A picture is projected into this aperture of a specially constructed lamp by an elliptic reflector (Philips' Gloeilampenfabrieken). This picture and the flash bulb are projected with a double condenser into the eye. A diffusion disc is necessary, but no cooling filter is used.

DESCRIPTION OF THE METHOD AND ITS THEORETIC BASIS

The optic-photographic system

Resolving power

The purpose of the method is to permit investigation of single and aggregated cells in the bloodstream. It is therefore desirable that the resolving power be sufficient to permit distinction between a single blood cell and a clump consisting of two erythrocytes or more. This requires a resolving power of at least 7 μ. The resolving power of the objective with oblique illumination of the area to be examined is given by the expression

$$d \cdot C_1 = \frac{\lambda}{2A}$$

where d = resolving power (distance between distinctly separated small elements)

λ = wavelength of light used

A = numerical of the objective

C_1 = constant ~ 1

The value of C_1 is determined by several factors, *e. g.* the obliquity of the light, the structure and the contrast of the field to be examined. The circumstances are relatively unfavorable when the bulbar conjunctiva is studied. If C_1 is taken as 0.5 (cp. Vogl: Instrumentaloptik) there is a good margin for the validity of the following calculations.

In direct microscopy and microphotography with panchromatic emulsions a light wavelength of 0.55 μ (yellow-green) can be used.

$$A > \frac{0.55}{7} \quad ; \qquad A > 0.08 .$$

The aperture should thus not be smaller than 0.08.

Depth of Focus

Photography of moving objects in high magnification requires a large-field view finder and good depth of focus. Movements that cannot be completely eliminated are blinking, physiologic nystagmus, arterial pulse, respiration, vibrations and those due to poor cooperation of the patient. The photographic field in this case is about 1 mm², so that even slight vibration will have a considerable disturbing effect.

A large-field view finder facilitates orientation. The area to be examined does not disappear from the field of vision even though it may disappear from the field to be photographed (see: *Camera arrangement).*

Great depth of focus facilitates fine adjustment and mitigates the effect of vibrations along the optical axis, the unevenness of the conjunctiva, and the convexity of the eyeball. Practical experiments have shown that with a depth of focus of less than 50 μ, focussing offers great difficulties.

Diameter of the vessels. The minimum acceptable depth of focus is dictated by the diameter of the vessels to be studied. Most of the vessels of relevant interest have a diameter of 7—60 μ. (The short arterio-venous anastomoses between arterioles and venules have a diameter of about 50 μ.)

The depth of focus should thus not be less than 60 μ.

The depth of focus is approximately inversely proportional to the square of the numerical aperture (Habell & Cox: Engineering Optics, page 150)

$$t = C_2 \cdot \frac{1}{A^2} .$$

where t = total depth of focus
A = numerical aperture of the objective
C_2 = constant.

The numerical aperture should thus be as small as possible in order to secure maximal depth of focus.

Microscope objectives with a working distance of more than 32 mm and with an aperture smaller than 0.1 can be regarded as thin lenses. (Cp. Engineering Optics page 142.)

The depth of focus can be calculated with the aid of the following formula which is approximately valid for thin lenses. (Formelheft des Optikers.)

$$t = \frac{2\,p\,(p-F)\,F^2\,z\,n}{F^4 - (p-F)^2\,z^2\,n^2} ;$$

where t = total depth of focus
p = distance between the principal plane of the objective and the focussing plane
F = focal length of the objective
n = diaphragm of the objective (= front opening of a thin lens)
z = distance between smallest separated details in the image.

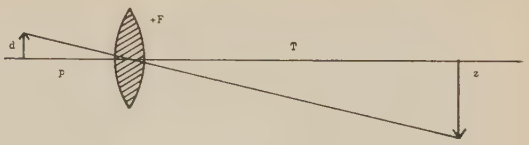

Fig. 1.

From Fig. 1 we get $z = \frac{Td}{p}$.

After combination of the formulas for t and z, application of the formula for the lens and simplification we get the following formula which is valid for a thin lens

$$t = \frac{d}{A} .$$

Choice of objective

After the calculations above it is possible to state that the numerical aperture of the objective must be 0.08 or slightly larger. The limits of the resolving power (7 μ) and depth of focus (60 μ) decide which objective is theoretically most suitable. These two limits must be balanced to give optimal results. Practical tests must then decide which objective gives the best results.

Table. *Comparison of the values for various numerical apertures, resolving power and depth of focus at various values of definition required.*

Numerical aperture	Resolving power in μ	Depth of focus in μ (greatest diameter of vessel) with varying degree (7 μ—5 μ) of utilisation of the resolving power			
		7 μ	6 μ	5.5 μ	5 μ
0.08	6.9	87	—	—	—
0.09	6.1	78	67	—	—
0.10	5.5	70	60	55	—
0.11	5.0	64	55	(50)	(45)
0.12	4.6	58	(50)	(46)	(42)

It is apparent from the table that objectives with a numerical aperture of 0.08—0.10 give the best results within the limits of the depth of focus and resolving power.

Total magnification

The blood cells, as they appear in the finished photograph, must be about 1 mm in diameter, *i. e.*, about 140 × to permit detection of erythrocyte aggregation. This size has been determined on purely empirical grounds.

A microscope objective filling the requirements discussed in the foregoing should have low magnification, *i.e.*, 2 × — 4 ×. All further magnification must be secured by the ocular and by enlargement of the negative. Enlargement must be kept within reasonable limits and not exceed that permitted by the size of the grains. A good fine grain emulsion (Adox KB 17) exposed with electronic flashlight and developed *lege artis* (Neodyn Red: 16 minutes at 20° C. in tank) can be enlarged tenfold. The purely optical primary magnification should, however, be utilised as far as possible to permit minimum enlargement.

The ocular should thus have as high a magnifying power as possible. Best correction is secured by a photo ocular with a magnification of 15 × — 20 ×.

The degree of magnification is also dependent on the photographic accessory. According to the definition of the degree of magnification of a microscope, the magnification on the film will be the same as the magnification by direct microscopy if the distance between the ocular and the plane of the film (referred to below as O — F distance) is 250 mm. If this distance is decreased, the magnification will be decreased to a corresponding extent. As a rule, the O — F distance is about 125 mm in ordinary camera accessories, so that the magnification is reduced by half, *i. e.*, a camera factor of 0.5. If the O — F distance is increased, the intensity of the light on the photographic film will be decreased (the intensity of light decreasing with the square of the distance). Therefore, a larger O — F distance is hardly recommendable. The theoretical magnification on the negative will thus be

obj.		ocular		camera factor	
4	×	15	×	0.5	= 30 times, —

with subsequent enlargement 150—300 ×.

Camera arrangement

As mentioned, the field of vision in the view finder should be large in relation to the field photographed. This facilitates focussing and orientation, because the flow of blood in the surroundings can then be observed during the exposure of the film. It is possible to recognise a characteristic area photographed on some earlier occasion and thereby to obtain comparable films.

In Zeiss-Winkel's accessory camera the field of vision of the view finder is five times as large as the area photographed when using 24 × 36 mm film. The camera factor is then 0.4, which corresponds to an O—F distance of 100 mm. If desired, this distance can be increased with a corresponding increase in the camera factor. Simultaneous exposure and observation is made possible by division of the pencil of light in a prism. The image in the adjustment eyepiece of the accessory camera is not good enough for examination, but it is sufficient for focussing and orientation.

Experimental apparatus

On the basis of these calculations the following optical system was used for the preliminary experiments.

Objective: Zeiss-Winkel Achromat 2.5 ×, focal distance 38 mm, numerical aperture 0.08. Resolving power about 7 μ.

Ocular: Zeiss-Winkel Photo 18 ×.

Camera factor: 0.5.

Magnification on negative: $2^1/_2 \times 18 \times 0.5 = 23$ times.

Total magnification: 230 ×.

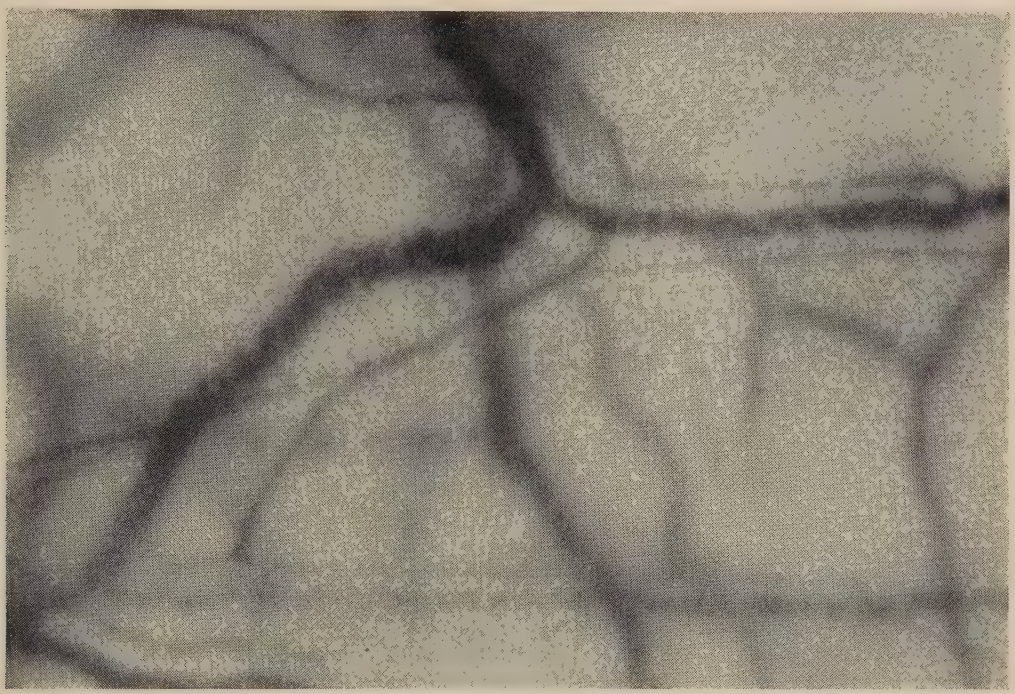

Fig. 2a. No aggregation. Magnification × 125.

It was found that the resolving power of the objective, which coincides with the theoretical minimum, was insufficient for more detailed investigation of the composition of the aggregates of erythrocytes but permitted satisfactory evaluation of their size. (Fig. 2.)

In later experiments we used the following system:

Objective: Bausch & Lomb Achromat 4 ×, focal distance 32 mm, numerical aperture 0.10. Resolving power about 5½ μ.
Ocular: Zeiss-Winkel Photo 18 ×.
Camera factor: 0.4—0.6.
Magnification on negative: 29—43 times.
Total magnification: 290 ×—430 ×.
(Fig. 3.)

Illumination

Exposure light

The source of exposure light consists of an electrone flash bulb. This has the following advantages:

a) The light is intense and can be adjusted for normal exposure of panchromatic films. Color photography is possible.

b) The exposure time is short and can be varied between 0.003 sec. and 0.00001 sec. If a blurring due to movement of the erythrocytes over a distance corresponding to one fifth of the diameter of a single erythrocyte can be tolerated (about 1½ μ) and the exposure time is 0.001 sec., the rate of flow must not exceed 1½ mm/sec.

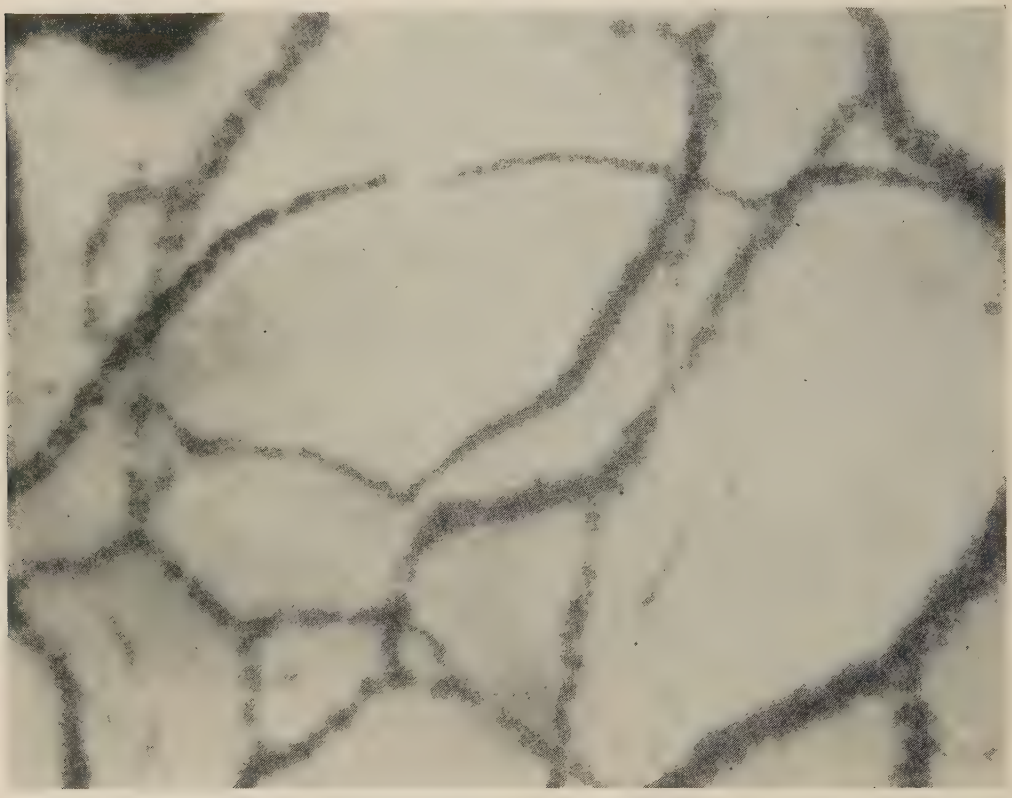

Fig. 2b. Moderate aggregation. Magnification × 160.

$$\text{Velocity} = \frac{1.5\ \mu}{0.001\ \text{sec.}} = \frac{1.5\ \text{mm}}{1\ \text{sec.}}$$

According to Ditzel, the following speeds have been recorded with a stopwatch and eyepiece micrometer for the circulation of the erythrocytes in man:

Arterioles	about 0.15 mm/sec.
Capillaries	about 0.03 mm/sec.
Venules	about 0.06 mm/sec.

c) Photographs can be taken in rapid succession. With the ordinary commercial electronic flash light accessories, up to ten exposures can be made per minute. With specially devised accessories this rate could surely be increased to 10 per second.

d) Experimental subjects are not inconvenienced by the light when exposures are taken every fifth second. The brief blink following an exposure is too late to influence the quality of the picture.

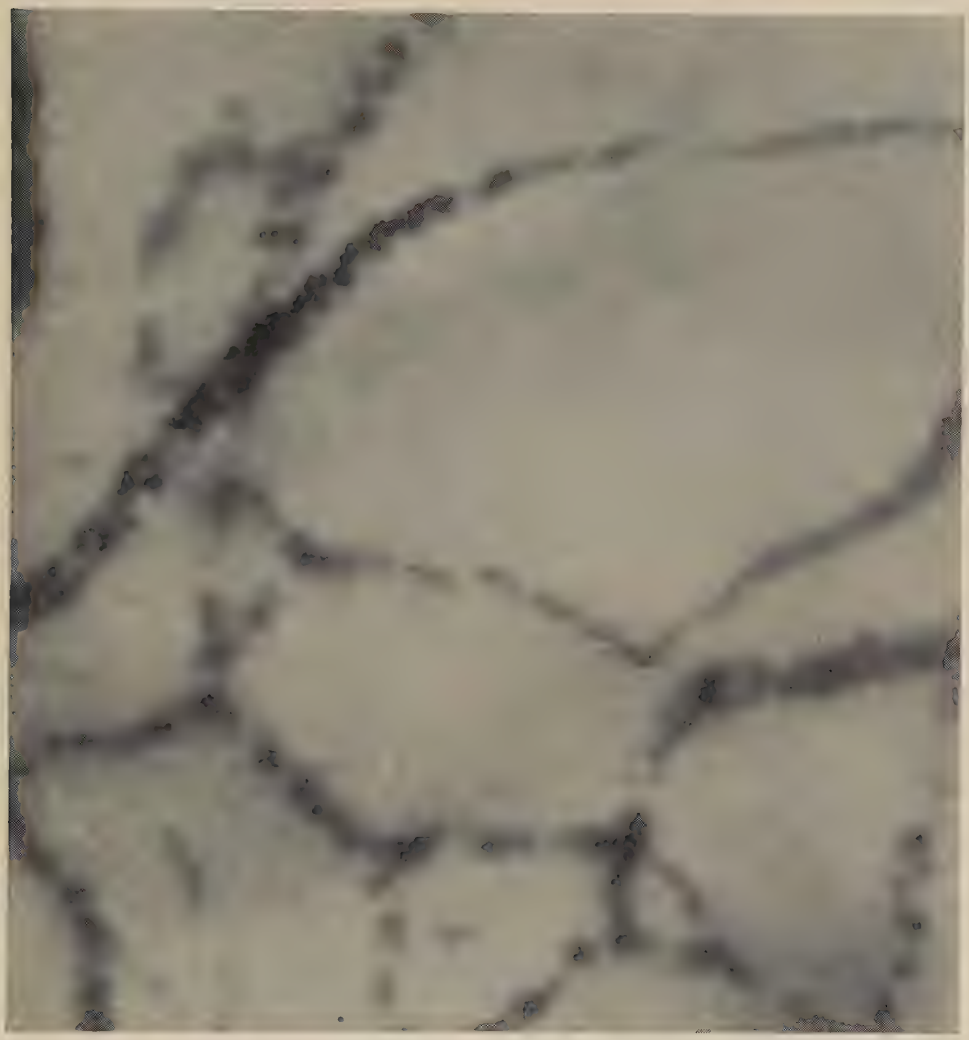

Fig. 2c. Detail of fig. 2b. Magnification × 250.

Pilot light

A Zeiss microscope lamp for oblique illumination gives a well defined, even field of light without disturbing side light. According to this principle the ratio between the condenser and projector lenses can be adjusted and the field of light thereby diminished *ad libitum*. A diameter of 5 mm is suitable.

A very small, point-like source of light is provided with a condenser. A relatively well corrected objective projects an image of the condenser onto the examination field. The size of the field can be adjusted with a diaphragm in or in front of the condenser. (Fig. 4.)

Fig. 3. No aggregation. Arrow indicates a single erythrocyte. Magnification × 250.

Avoidance of reflexes by combination of the exposure light and the pilot light in one and the same appliance

In all attempts hitherto made to photograph the eye, reflexes have been disturbing. These reflexes were due to the difference in the angle of incidence of the pilot light and of the exposure light. It is not possible to predict where reflexes of the exposure light will arise unless they can be made to coincide with those of the pilot light.

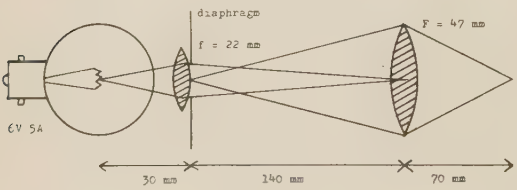

Fig. 4.

In the first experiments the pilot light was placed immediately before the flash tube with its reflector. The minute loss of light was negligible, but diffuse light from the reflector still gave uncontrollable reflexes. (We therefore intended to manage without the flash bulb reflector and place the pilot light behind the flash bulb, but we were unable to obtain a suitable flash tube. (Cp.Winkelman & Warmoltz, 1954.)) In order to avoid reflexes we devised a special lamp containing both sources of light. The electrone flash and the condenser of the pilot light were positioned so that they could be projected onto the same spot through a common objective. A piece of thin plane glass was mounted between the electrone flash bulb and the objective, at 45 degrees to its axis. The light from the pilot lamp was reflected by this plane glass into the objective. Then both sources of light gave exactly the same reflexes which could easily be avoided at photography. Finally, the lamp was provided with an effective cooling filter (Zeiss, Jena BG 21), which eliminated all invisible light without any appreciable decrease in the strength of the light (Fig. 5).

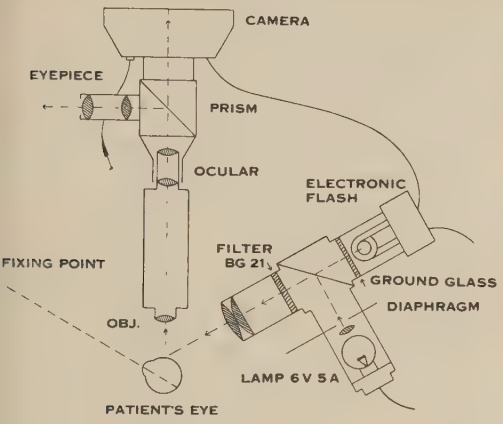

Fig. 5.

With this lamp the subject is not inconvenienced, not even when examined for as long as minutes. Even children 4—6 years of age have been examined without difficulty.

Practical application of the method

Thanks to the low magnifying power of the objective the working distance is long. This implies that the subject has not the impression that the eye is unprotected from the apparatus. Experimental animals (rabbits and dogs) can therefore be examined without being frightened. Should the patient or the experimental animal jerk on discharge of the flash, there will be no risk of injury to the eye.

The patient is preferably examined in a supine position with the head supported in a ring. This ring can be attached to the side of the bed or examination table or be placed on the pillow if the patient cannot be transported. The apparatus can also be used without difficulty during an operation. With the patient in a supine position the head can be turned to a suitable angle, after which the patient is requested to fix his eyes on

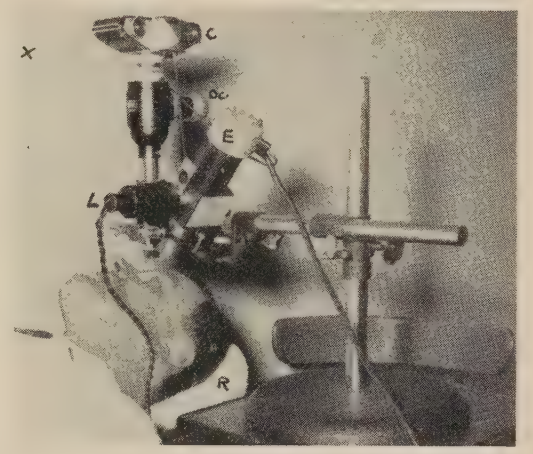

Fig. 6. The complete appliance in clinical use. C, camera; E, electronic flashtube; L, lamp; Oc, ocular of view finder; R, ring for patient's head; ×, fixing point.

a small lamp or some other stationary object so that the eye may assume a suitable position.

SUMMARY

A description is given of a method for microphotography of the vessels in the conjunctiva bulbi. The method, which is atraumatic, can be used for continuous and comparative studies on vessels and circulating erythrocytes in areas of the magnitude of 1 mm². The magnification on the photographic negative is 25—40 times and the final magnification is about ten times larger.

Optimal conditions for good reproduction are calculated on purely physical grounds with respect to resolving power, depth of focus, desired magnification, exposure light and pilot light.

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STATISTICAL STUDIES ON THE ERYTHROCYTE SEDIMENTATION RATE OF HEALTHY YOUNG ADULTS

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The erythrocyte sedimentation rate (ESR) has become etasblished as one of the most important laboratory tests for the presence of inflammatory and also some other disease processes. On the other hand, less interest has been devoted to analysing the variations of ESR within the normal range. The pertinent literature has been reviewed *e. g.* by Nichols (1942).

It has been claimed that the normal ESR shows typical diurnal variations (Jores & Strutz, 1936), but contrary results have also been published (Renbourn, 1947). A sex difference is well known, women generally having a higher ESR than men. The sex difference disappears after the age of 50. The ESR of apparently healthy people rises with advancing age until about 70 years, after which it remains fairly constant. The change with ageing is smallest in individuals who appear fittest. With age, the scatter of the ESR also increases (Renbourn & Ellison, 1952).

Normal subjects on whom repeated ESR determinations have been made often claim that they generally have a "low" or a "high" ESR. It has been observed that an ESR of 2—6 mm in the morning may rise to 10—15 mm by the afternoon or evening in perfectly normal individuals (van Antwerp, 1934; Renbourn, 1947). Nevertheless, when ESR determinations have been made a week apart, it has been shown that healthy individuals have their own characteristic levels of ESR as well as of hemoglobin, Hb) and plasma protein; the inter-individual variation is statistically significant (Renbourn). This gives a statistical confirmation to earlier qualitative statements of the individual constancy of the ESR (*e. g.* Greisheimer, 1927; Wintrobe & Landsberg, 1935; Smith 1936).

The purpose of the present study was first to extend the above study of individual constancy over a longer period, about 4½ months, also using a larger material, both men and women. The study was made to cover the 2 hr. and 24 hr. ESR in addition to the usual 1 hr. reading, and the blood Hb level.

Second, the problem was posed, whether healthy groups of about the same age might show significant differences; in other words, whether ESR or Hb would have any value as group characteristics among healthy subjects of the same age and sex. The effect of season on ESR and Hb was also studied.

Third, the intercorrelations of 1 hr., 2 hr. and 24 hr. ESR and of the Hb were calculated separately for men and women. The effect of season on the intercorrelations was also determined.

MATERIAL AND METHODS

The subjects of the study were 71 male and 63 female students of the Helsinki University School of Physical Education. The range of their ages was from 18 to 23 years. The students, whose studies last for three years, were correspondingly grouped into three classes. Blood samples were taken from them first between September 15 and October 15, 1953, and again during February 1954. Thee ESR was determined in Westergren's tubes, in a vertical column of blood 200 mm high. 1.6 ml of blood was mixed with 0.4 ml of 3.8 per cent sodium citrate (Westergren, 1924). The racks of tubes were kept at a constant temperature, + 22—23° C.

Hb was determined from capillary blood, as acid hematin, using a photoelectric colorimeter (Klett-Summerson, Klett Mfg. Co., N. Y., U. S. A.).

In the statistical analysis, current methods of regression and variance analysis were used *(e. g.* Fisher, 1950).

RESULTS

Individual constancy. The individual constancy of the ESR and Hb values was determined by calculating the correlation coefficient between the results of the first and second determination. The results are shown separately for men and women in Table I. Instead of the correlation coefficient, r, the term $(1-\sqrt{1-r^2}) \times 100$ is tabulated; the latter has the advantage of directly showing the percentage share of variation explained by the observed correlation. The term will be called "efficiency percentage".

Table I. *The intra-individual constancy of the ESR and Hb between first and second determinations.*

Variable	$(1-\sqrt{1-r^2})\times 100$	
	Men, n=71	Women, n=63
ESR: 1 hr.	23	12
2 hrs.	22	11
24 hrs.	13	6
Hb	15	5

Correl. signific. at the P<0.05 level: Light underl.
» » » » P<0.01 » Semi-bold »
» » » » P<0.001 » Bold »

In all measurements examined the intra-individual correlation was stricter in men than in women. However, the difference between men and women of none of the correlations attains statistical significance. Nevertheless, since all of them differ in the same direction, this suggests that in these measures the men show more constancy than the women.

In both sexes the individual constancy is most marked with the 1 hr. ESR and weakest with Hb.

ESR and Hb as group characteristics. Table II lists the mean values of the observations for each group and for each determination. The corresponding standard deviations are shown in Table III.

In order to find out which of the observed differences between the various groups were significant, a variance analysis was made. The results of the variance analysis are shown in Tables IV—VII.

Several significant differences appeared. Those between the classes were studied further, by splitting the variance between classes in two parts. The results of this continuation of the variance analysis are shown in Table VIII.

All the measures examined showed a significant *sex* difference, the ESR being higher in women, and the Hb level higher in men. Significant *seasonal* differences were observed in the 24 hr. ESR in both sexes and in the Hb level of men.

Table II. *The mean values of the ESR and Hb for each group and determination. A = autumn, W = winter.*

Group			n	ESR mm. 1 hr. A	ESR mm. 1 hr. W	ESR mm. 2 hrs. A	ESR mm. 2 hrs. W	ESR mm. 24 hrs. A	ESR mm. 24 hrs. W	Hb g/100 ml A	Hb g/100 ml W
Men:	Class	I	25	2.7	3.5	7.9	10.2	57.9	64.8	15.67	15.07
	Class	II	23	4.5	4.5	12.3	14.0	57.0	69.6	15.60	14.85
	Class	III	23	4.7	5.0	11.8	13.3	58.3	74.0	15.62	15.13
	Comb.	I-III	71	3.9	4.3	10.6	12.4	57.7	69.3	15.63	15.02
Women:	Class	I	22	10.0	9.0	25.9	23.1	94.9	95.8	13.26	12.96
	Class	II	20	6.6	7.4	17.3	20.1	74.9	90.6	13.56	14.31
	Class	III	21	6.8	8.7	17.5	21.4	69.4	87.8	13.59	12.59
	Comb.	I-III	63	7.8	8.4	20.4	21.6	80.0	91.5	13.46	13.26

The 24 hr. ESR was lower in the autumn than in the winter, and the Hb of men was lower in winter than in autumn.

Significant *class* differences were observed in all measures. The 1 hr. *ESR of the men* of Class I was lower than that of the men of Class II and III. In the 2 hr. ESR, Class I differed significantly only from Class II, and in the 24 hr. ESR men showed no class differences.

Among the *women* Class I regularly had the highest ESR values. In the 1 and 2 hr. ESR the women of Class II had the lowest mean values, but in the 24 hr. ESR Class III was lowest. The 24 hr. ESR showed a significant class/season interaction, evidently caused by the increase of the values of Class II and Class III from autumn to winter.

A significant class difference and a class/season interaction were observed in the *Hb values of women.* Class I had a lower Hb level than Class II. The class/season interaction is evidently due to a marked fall of the Hb of Class III from autumn to winter.

Intercorrelations of ESR and Hb. The values obtained were also used for determining the interdependence of the ESR readings after 1, 2 and 24 hrs., and of the blood Hb concentration. The results are shown in Table IX. The 1 and 2 hr. ESR are rather closely correlated. On the other hand, the interdependence of ESR and Hb is markedly low, though significant. In general, the different measures are more closely correlated with each other in men than in women.

Effect of season and class on the intercorrelations. The various intercorrelations were also calculated separately for each class and season. The numerical value of the correlation coefficient often varied markedly from class to class and from autumn to winter, but none of these differences proved statistically significant. Therefore, only the pooled correlations are given.

Table III. *The standard deviations of the ESR and Hb for each group and determination. A = autumn, W = winter.*

Group		n	ESR mm. 1 hr. A	ESR mm. 1 hr. W	ESR mm. 2 hrs. A	ESR mm. 2 hrs. W	ESR mm. 24 hrs. A	ESR mm. 24 hrs. W	Hb g/100 ml A	Hb g/100 ml W
Men:	Class I	25	2.2	2.0	5.9	6.0	23.5	13.8	1.06	1.26
	Class II	23	3.0	3.6	8.4	9.7	25.4	20.6	1.28	1.78
	Class III	23	3.6	2.7	8.9	6.9	27.4	15.7	0.86	1.23
	Comb. I-III	71	3.1	2.9	7.9	7.7	24.9	17.0	1.06	1.41
Women:	Class I	22	4.6	2.8	10.1	6.9	11.7	8.7	1.29	1.45
	Class II	20	3.4	4.5	8.6	12.9	21.6	15.4	1.05	1.26
	Class III	21	4.8	5.4	12.1	11.7	29.6	15.0	0.59	1.25
	Comb. I-III	63	4.5	4.3	11.0	10.5	24.4	13.5	1.01	1.50

DISCUSSION

Some of the characteristics of man are obviously very stable, *e. g.* the anthropometric measures determined by the bones. On the other hand, the results of many physiological and chemical measurements are known to vary with time. The *individual constancy* of such measures may be more or less pronounced. Plasma alkaline phosphatase (Clark & Beck, 1950), serum pseudocholinesterase and serum cholesterol are examples of relatively unchanging chemical characteristics of the individual (Tanner, 1955), whereas *e. g.* the composition of urine probably shows very little individual constancy.

The present results show that even in healthy young adults the ESR and Hb showed statistically significant test-retest correlations over a period of about 4½ months (Table I). The individual constancy explains from 5 to 23 per cent of the observed variation of these values.

It is interesting to note that the individual constancy tended to be lower in women than in men. The same observation has been made previously (Greisheimer, 1927; Wintrobe & Landsberg, 1935) though expressed only in qualitative terms. The more pronounced lability of women may partly depend on the sexual cycle, though no significant changes with menstrual period have been observed (Greisheimer, 1927; Wintrobe & Landsberg, 1935). On the other hand, it may also be an expression of a general phenomenon: the female organism is more "plastic" than the male. The individual constancy of the electrolyte concentration of thermal sweat, for instance, shows a similar sex difference (Karvonen, 1956). *"La donna e mobile".*

The values of ESR obtained in the present study (Table II) closely agree with those

Table IV. *Analysis of variance, ESR, 1 hr.*

Source of variation		Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Between sexes		1060	1	1060.00	76.98	0.001
Within males:	between seasons	7	1	7.00	(1.22)*	..**
	between classes	86	2	43.00	5.05	0.01
	class/season interaction	2	2	1.00	(8.52)	..
	residual	1159	136	8.52	.	.
Within females:	between seasons	9	1	9.00	(2.05)	..
	between classes	142	2	71.00	3.84	0.05
	class/season interaction	41	2	20.50	1.11	..
	residual	2218	120	18.48	.	.
Total		4724	267	.	.	.
thereof: total within sexes		3664	266	13.77	.	.

* Values in brackets are inverted values

** .. means P>0.05

reported by other investigators on healthy young adults (*e. g.* Wintrobe and Landsberg, 1935: mean of men 3.7 mm, of women 9.6 mm, Terry (1950): men 3.8 mm, women 4.7 mm. Gallagher's (1934) 1 hr. mean of boys was 4.7 mm and 2 hr. mean 9.7 mm).

It has previously been shown that the ESR of normal women shows more inter-individual variation than that of men(*e. g.* Wintrobe & Landsberg, 1935). An examination of the standard deviations (Table III) shows that such a tendency exists; on the other hand, no sex difference in the scatter of the Hb values is observable.

The differences between the *seasons* and the *classes* obviously have no general applicability; they reflect the particular conditions under which the subjects were living. However, they are presented here in order to demonstrate how sensitive these measurements are to indicate differences even between groups working and living under closely similar conditions. Their interpretation is left to speculation. The Hb level of women was evidently more prone than that of men to change under the influence of university life, whether it be through the activities imposed by the curriculum, or through their extracurricular mode of life, like eating habits. The girls of Class II throughout showed the highest Hb level; this is the year in which the girls also have most physical exercise. This finding is in variance with the results of Adcock, Brown, Kerr, Magee & Milligan (1948) who found a higher Hb concentration in sedentary than in corresponding "active" groups of men.

The contribution of eventual short-term influences must also be considered in interpreting both Hb and ESR changes. Thus, ESR has been reported to change under the effect of meteorologic changes (Hoverson & Petersen, 1934), and exercise has been claimed to cause both rises and falls of the

Table V. *Analysis of variance, ESR, 2 hrs.*

Source of variation		Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Between sexes		5976	1	5976.00	68.90	0.01
Within males:	between seasons	119	1	119.00	2.00	..**
	between classes	471	2	235.50	3.95	0.05
	class/season interaction	5	2	2.50	(23.85)*	0.05
	residual	8108	136	59.62	.	.
Within females:	between seasons	46	1	46.00	(2.39)	..
	between classes	856	2	428.0	3.90	0.05
	class/season interaction	281	2	140.50	1.28	..
	residual	13.185	120	109.88	.	.
Total		29.047	267	108.79	.	.
Thereof: total within sexes		23.071	266	86.73	.	.

* Values in brackets are inverted values
**..means P>0.05

Table VI. *Analysis of variance, ESR, 24 hrs.*

Source of variation		Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Between sexes		33069	1	33069.00	72.30	0.01
Within males:	between seasons	4770	1	4770.00	10.22	0.01
	between classes	563	2	281.50	(1.66)*	..**
	class/season interaction	482	2	241.00	(1.94)	..
	residual	63442	136	466.49	.	.
Within females:	between seasons	4125	1	4125.00	12.44	0.01
	between classes	6206	2	3103.00	9.36	0.01
	class/season interaction	2268	2	1134.00	3.42	0.05
	residual	39802	120	331.68	.	.
Total		154727	267	579.50	.	.
Thereof: total within sexes		121658	266	457.36	.	.

* Values in brackets are inverted values

** .. means P>0.05

Table VII. *Analysis of variance, Hb. Numerical values refer to Hb percentages.*

Source of variation		Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Between sexes		10550	1	10550.00	155.70	0.01
Within males:	between seasons	553	1	553.00	8.26	0.01
	between classes	31	2	15.50	(4.32)*	..**
	class/season interaction	15	2	7.50	(8.93)	..
	residual	9104	136	66.94	.	.
Within females:	between seasons	50	1	50.00	(1.14)	..
	between classes	757	2	378.50	6.62	0.01
	class/season interaction	654	2	327.00	5.72	0.01
	residual	6860	120	57.17	.	.
Total		28574	267	107.02	.	.
Thereof: total within sexes		9703	141	68.82	.	

* Values in brackets are inverted values

** .. means P$>$0.05

Table VIII. *Analysis of variance. Splitting of the variation between classes to single degrees of freedom.*

	Comparison	Sum of squares	Degrees of freedom	Variance	Variance ratio	P
ESR, 1 hr.	Males I/II	48	1	48.00	5.63	0.05
	Males I-II/III	38	1	38.00	4.46	0.05
	Females I/II	143	1	143.00	7.74	0.01
	Females I-II/III	8	1	8.00	(2.31)*	..**
ERS, 2 hrs.	Males I-II	401	1	401.00	6.73	0.05
	Males I-II/III	70	1	70.00	1.17	..
	Females I/II	711	1	711.00	6.47	0.05
	Females I-II/III	145	1	145.00	1.32	..
ESR, 24 hrs.	Males I/II	90	1	90.00	(5.18)	..
	Males I-II/III	470	1	470.00	1.01	..
	Females I/II	2984	1	2984.00	9.00	0.01
	Females I-II/III	3222	1	3222.00	9.71	0.01
Hb	Males I/II	24	1	24.00	(2.79)	..
	Males I-II/III	7	1	7.00	(9.56)	..
	Females I/II	559	1	559.00	9.78	0.01
	Females I-II/III	95	1	95.00	1.66	..

* Values in brackets are inverted values

** .. means P$>$0.05

Hb level, depending on its intensity. Westergren showed as early as in 1924 that several forms of stress, after a latent period, typically cause, a rapid increase of the ESR, and subsequently a slow decrease.

In comparing groups of normal subjects, the ESR must therefore be recorded under strictly controlled conditions. The present results show that the ESR of groups of subjects are significantly influenced by conditions common to the group; thus, variations in the normal ESR may be considered a promising subject for further study.

Wintrobe & Landsberg found in young adult men a negative *correlation* between the 1 hr. *ESR* and *hematocrit*, $r = -0.233$, but no correlation in women, $r = -0.026$. Renbourn's and Ellison's correlation between the ESR and hematocrit value was: $r = -0.270$, $P < 0.05$. In the present series the correlation coefficient between 1 hr. ESR and Hb level was in men $r = -0.25$, $P < 0.01$ and in women $r = -0.30$, $P < 0.01$. If subjects with anemia or higher ESR had been included, the correlations might have been higher. Thus, Lucia *et al.* (1936) observed a correlation of $r = +0.41$ between erythrocyte count and sedimentation time in 102 pathological cases.

Among the intercorrelations between the various measurements, only those between 1 hr. and 2 hr. ESR were sufficiently high to have any predictive value among normal subjects. Again, when working with pathological cases and a wider range of ESR, the intercorrelations may become quite different. For instance, a high negative correlation is to be expected between 24 hr. ESR and Hb with high sedimentation rates.

Table IX. *Intercorrelation of the ESR readings after 1, 2 and 24 hrs., and of the blood Hb concentration. Correlations expressed as* $(1 - \sqrt{1-r^2}) \times 100$. *Significance expressed as in Table I. The signs + and — refer to the sign of r.*

		ESR, men			Hb
		1 hr.	2 hrs.	24 hrs.	men
ESR, women	1 hr.		+72	+26	—3
	2 hrs.	+63		+31	—3
	24 hrs.	+17	+21		—6
Hb women		—5	—3	—1	

Ultimately, ESR depends to an essential extent on the composition of the plasma protein (Westergren, Theorell & Widström, 1931). Individual characteristics of the ESR and its changes are thus indicators of corresponding phenomena in the metabolism of the plasma proteins.

SUMMARY

The individual constancy over a period of 4½ months of 1 hr., 2 hr., and 24 hr. erythrocyte sedimentation rate (ESR) and of blood hemoglobin concentration (Hb) was studied in healthy young adult male and female students. All measures showed a significant intra-individual constancy; this explained from 5 to 23 per cent ("efficiency percentage" $(1 - \sqrt{1-r^2}) \times 100$) of the variation observed. The values for men were

systematically more constant than those for women.

When the subjects were grouped according to their sex and study programs, significant group and seasonal differences appeared. The interpretation of these was left open. The existence of such differences demonstrates that the changes of ESR and Hb within the normal range may be used as a tool in studying the effects of environmental factors on groups.

The intercorrelations between the different forms of ESR explained from 17 to 72 per cent of the variations. The correlations between Hb and ESR were considerably lower, explaining only from 1 to 6 per cent of the variations.

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THE THROMBIN GENERATION TEST IN THE DIAGNOSIS OF CLASSICAL HEMOPHILIA AND CHRISTMAS DISEASE

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The prothrombin consumption test described by Quick (1947, 1949) has been a useful tool in the diagnosis of hemophilia. In principle this method measures the disappearance of prothrombin in serum after the spontaneous clotting of blood. The disappearance of prothrombin is a measure of its transformation into thrombin and gives an indication of the velocity of the thrombin formation. Consequently it can be used as an indication of the activity in blood of the systems involved in the conversion of prothrombin into thrombin. Provided that the concentrations of prothrombin as well as of proaccelerin (factor V) and proconvertin (factor VII) in the blood sample are normal, and if also the inhibitory agents are normal, the results provide an expression of the activity of the plasma thromboplastin system.

However, the formation of thrombin can be followed directly by suitable methods (Macfarlane & Biggs, 1953; Pitney & Dacie, 1953) instead of indirectly by the disappearence of prothrombin. Theoretical and practical reasons make estimation of the thrombin generation a better method for estimating the activity of the activating complex (plasma thromboplastin) involved in the conversion of prothrombin.

Normally prothrombin is present in high concentrations in blood. This makes the estimation of a small decrease in prothrombin concentration very inaccurate, because it appears as a small difference between two large quantities of prothrombin. Consequently an almost complete transformation of prothrombin is essential for this test, and the process has to be followed for hours, in order to estimate its end point.

On the other hand the formation of even small amounts of thrombin is easily observed. This makes it possible to follow the initial phase of the thrombin formation, where a rapid increase in thrombin concentration occurs. This is of importance also because a short reaction period makes it easier to keep constant all other conditions involved in the process. An objection of practical nature to the routine use of the prothrombin consumption test is that it can be performed only at the bedside.

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In preliminary investigations on citrated plasma from hemophilic patients, the thrombin generation test had shown its usefulness and superiority over the prothrombin consumption test (Astrup, 1954, personal communication). Therefore, a detailed investigation of the thrombin generation test was taken up in order to study its applicability in the differential diagnosis of the various forms of hemophilia.

MATERIALS AND METHODS

The following reagents were used:

Saline: 0.85 per cent NaCl.

Sodium citrate: 3.8 per cent in water.

Calcium chloride solution: 0.025-M $CaCl_2 \cdot 6\ H_2O$.

Saline diethylbarbiturate buffer: sodium diethylbarbiturate buffer (Michaelis), 0.05 M, pH 7.8, containing 0.10 M NaCl. Total $\mu = 0.15$.

Adsorbed bovine plasma. Treated twice with 2 g of $BaSO_4$ per 100 ml according to Brodthagen (1953).

Bovine fibrinogen (Astrup & Müllertz, 1952) diluted immediately before use to 0.15 per cent fibrinogen with saline diethylbarbiturate buffer.

Samples of test plasma used in cross matching were centrifuged for 15 mins. at 2500 r. p. m. and stored at $-20°$.

Test tubes and pipettes were cleaned with sodium carbonate and dichromate-sulfuric acid.

Thrombin generation test:

4.5 ml blood was run directly from the cannula in the vein into 0.5 ml sodium citrate solution in a silicone coated glass tube and mixed by gentle inversion of the tube. The citrated blood was centrifuged sligthly for two minutes at a maximum speed of 1000 r. p. m. and the plasma separated. In each of 13 glass tubes of uniform size (internal diameter 8.5 mm, no silicone) were placed 0.4 ml fibrinogen solution. The tubes were placed in a rack in a water bath at 37° C. In another test tube 1 ml of the plasma sample and 1 ml saline were mixed, and the mixture was allowed to reach the temperature of the water bath. Then 1 ml of pre-heated (37° C) calcium chloride solution was rapidly added. The contents of the tube were shaken and a stopwatch started. Beginning at 1 minute after recalcification and then at one minute intervals, 0.1 ml of the reaction mixture was transferred with a micropipette to successive fibrinogen tubes, and clotting times (t) of the fibrinogen solutions in these tubes were recorded. At a time which is the recalcification time of the diluted plasma, the reacting mixture clotted. This time was noted. The clot was pressed against the wall of the test tube with two wooden sticks during the rest of the procedure.

A curve relating the clotting time (t) of the fibrinogen solution to the reaction time (T) of the plasma sample was prepared for each sample. An arbitrary unit of thrombin was chosen for the purpose of comparing one experiment with another. This unit was given as the reciprocal value of the clotting time t of the fibrinogen solution, measured in seconds, and calculated as 600/t. The concentration of thrombin is inversely proportional to the clotting time of fibrinogen (Astrup & Darling 1942). Two thrombin generation tests on normal plasma were performed daily as controls. The Quick prothrombin time vas estimated according to Biggs & Macfarlane (1953) with human brain thromboplastin (Owren, 1949). Prothrombin-proconvertin was estimated with Owren's P-P-method (Owren, 1949; Astrup, Müllertz & Hansen, 1951).

RESULTS

In the following some results of the application of the thrombin generation test to normal and hemophilic blood are reported, with special reference to the differential diagnosis between classical hemophilia and Christmas disease.

The thrombin generation in *normal plasma* is shown graphically in Fig. 1. Plasma samples from 8 persons with no clinical signs of hemorrhagic disorders were used. The Quick prothrombin time was normal in all the samples. It appears from the figures that

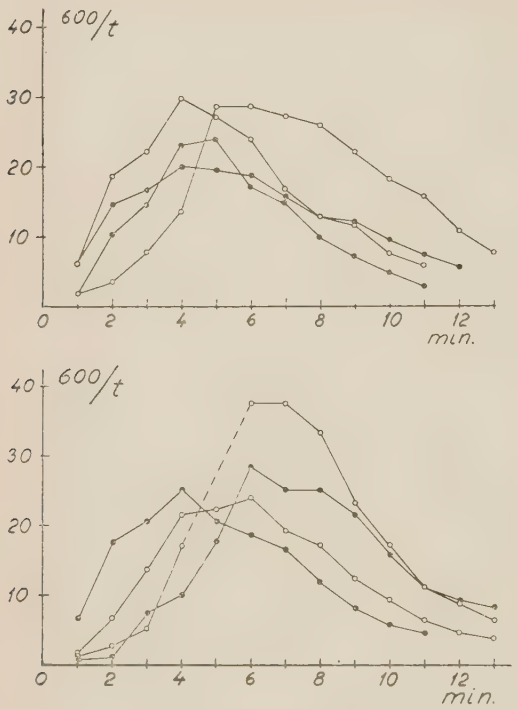

Fig. 1. Thrombin generation in normal plasma. *Abscissa:* Reaction time (T) in minutes. *Ordinate:* Reciprocal clotting time expressed as 600/t (t in seconds).

the thrombin formation is slow during the first two minutes after recalcification. After this lag period the thrombin concentration increases quickly and reaches its maximum 4—7 minutes after recalcification. Thereafter the thrombin is rapidly inactivated, and after 10—12 minutes of incubation the thrombin concentration is very low.

In *hemophilic plasma* the formation of thrombin is very slow. It should be expected that the poor thrombin generation in classical hemophilia (AHF deficiency) would become normal by addition of small amounts of antihemophilic globulin such as contained in barium sulphate treated bovine plasma, citrate plasma from a patient with Christmas disease or normal citrate plasma, whereas the addition of normal serum or of plasma from another patient with classical hemophilia should be without effect.

In *Christmas disease* the poor thrombin formation should be improved by addition of small amounts of serum or plasma from normal persons as well as from patients with classical hemophilia (AHF deficiency). Barium sulphate adsorbed bovine plasma or plasma from another patient with Christmas disease should be without effect on the thrombin generation .

Experiments performed to test these assumptions are recorded below together with the appropriate clinical data. Some of the patients investigated belong to the families studied by Andreassen (1943), and the family numbers used by him are retained and continued.

1. *Family 14* (Andreassen, 1943). Male born 1938. Fig. 2. 16 year old patient with hemorrhagic disorder from the age of 6 months. Bleeding from the gums, hemarthroses followed by joint contractures, muscular hemorrhages and one intra-abdominal bleeding followed by sensibility disturbances in the left leg. Laboratory investigations: Quick "prothrombin" 84—100 per cent. Prothrombin-proconvertin *a. m.* Owren 100 per cent. Recalcification time of diluted plasma 7 minutes (controls: 1½— 4 minutes). The thrombin generation was very poor and the thrombin concentration never reached values comparable with normal values (Fig. 2a). Addition of 0.2 ml absorbed bovine plasma, or 0.2 ml citrated plasma from a patient with Christmas disease did not improve the thrombin generation (Fig. 2e and 2c), whereas an addition of 0.2 ml thrombin free serum (Biggs & Macfarlane, 1953) or 0.2 ml plasma from a patient with classical hemophilia made the thrombin generation normal (Fig. 2d and 2b).

Diagnosis: Christmas disease.

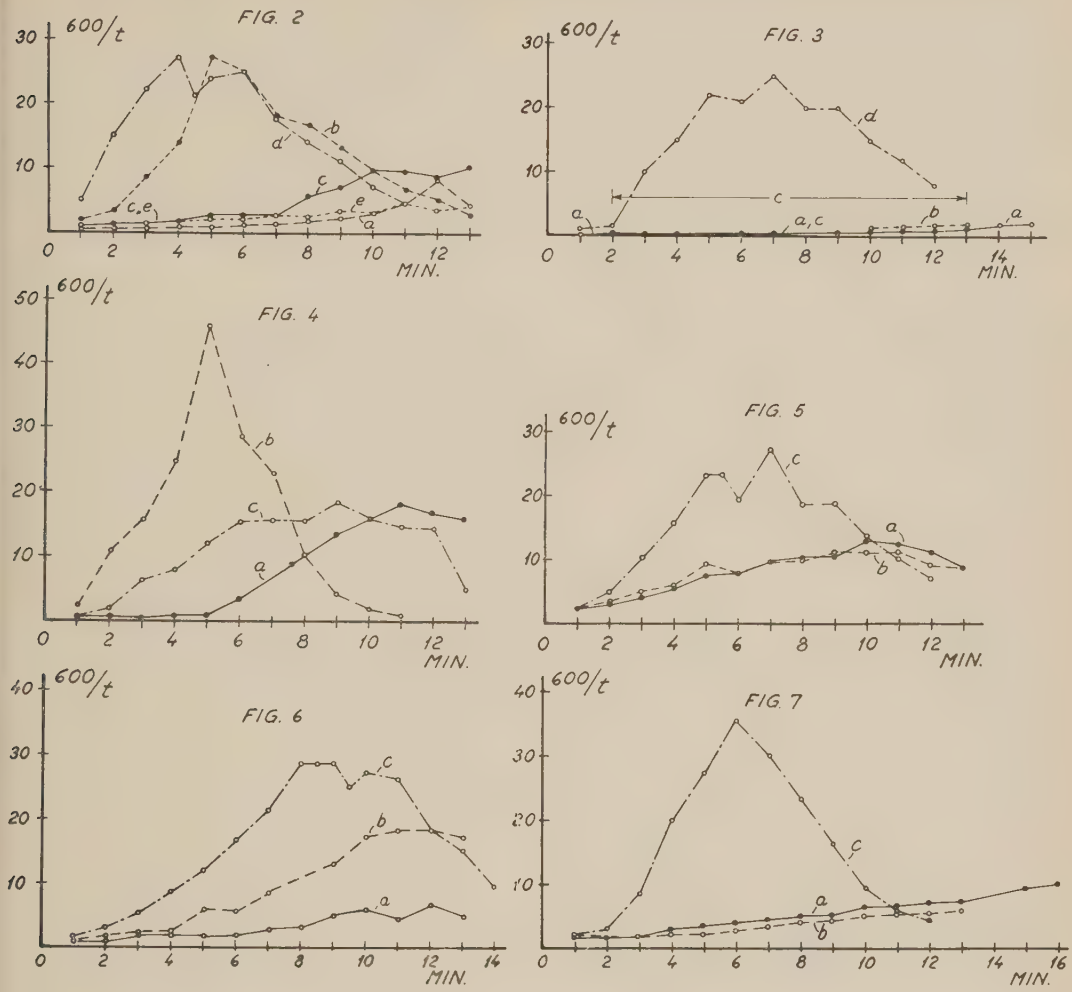

Fig. 2—7. Thrombin generation in plasma from plasma with classical hemophila or Christmas disease. Values recorded as in Fig. 1.
For details see text.

2. *Family 4* (Andreassen, 1943). Male born 1896. Fig. 3. 61 year old man with bleeding tendency from the age of 18 months. Multiple hemarthroses followed by regressiv joint lesions. Often hematuria. Male relatives with hemorrhagic disorders.

Laboratory investigations: Quick "prothrombin" 115 per cent. Recalcification time of diluted plasma: 12 minutes. In the thrombin generation test hardly any thrombin formation was observed during 15 minutes (Fig. 3a). No improvement on addition of 0.2 ml absorbed bovine plasma or of 0.2 ml citrated plasma from one of his affected relatives (Fig. 3b and 3c). On addition of 0.2 ml citrate plasma from a patient with AHF deficiency the thrombin generation became normal (Fig. 3d).

Diagnosis: Christmas disease.

3. *Family 23* (Andreassen, 1943). Male born 1950. Fig. 4 shows the thrombin generation tests in plasma from a 5 year old boy. From the age of 18 months several subcutaneous bleedings, recurring hemorrhages in elbow and ankle joints. Several cases of bleeding tendency among male relatives.

Laboratory investigations: Recalcification time on diluted plasma 6′45″. The thrombin generation shows a prolonged lag period and begins at about 6 minutes after recalcification. Larger thrombin concentrations were first measured after 11 minutes (Fig. 4 a). On addition of 0.2 ml citrate plasma from a patient with AHF deficiency, the thrombin concentration became maximal after 5 minutes (Fig. 4b). An addition of adsorbed bovine plasma (adsorbed only once) had only a slight effect (Fig. 4 c).

Diagnosis: Christmas disease.

4. *Family 71.* Male born 1949. Fig. 5 shows the thrombin generation in plasma from a 6 year old boy, who from the age of one year has had several bleedings in the elbow joints and in the subcutaneous tissue. There are other males with bleeding tendency in the family.

Laboratory investigations: Clotting time on blood (37° C) 3½ hours. Bleeding time 4½—8 minutes. Recalcification time on diluted plasma 4½ minutes. Quick "prothrombin" 96 per cent. The thrombin developed very slowly and attained very low concentrations (Fig. 5a). The addition of 0.2 ml thrombin-free serum from a patient with classical hemophilia (AHF deficiency) was ineffective (Fig. 5b). On addition og 0.2 ml bovine plasma the thrombin generation became normal (Fig. 5c).

Diagnosis: Classical hemophilia (AHF deficiency).

5. *Family 2* (Andreassen, 1943). Male born 1922. Fig. 6. 33 year old man, who from earliest childhood has suffered from recurring hemorrhages in joints, in subcutaneous tissues and from the kidneys. Several male relatives show bleeding tendency.

Laboratory investigations: Quick "prothrombin" 110 per cent, prothrombin *a. m.* Owren 100 per cent. Recalcification time of diluted plasma 10 minutes. Clotting time *a. m.* Bürker-Andreassen 27 minutes. The formation of thrombin was very slow and had not reached noticeable values after 13 minutes (Fig. 6a). On addition of 0.2 ml citrated plasma from a patient with classical hemophilia, the thrombin generation improved slightly (Fig. 6 b). The addition of 0.2 ml bovine plasma improved the thrombin generation considerably (Fig. 6c). At the same time the recalcification time decreased to 1½ minutes.

Diagnosis: Classical hemophilia (AHF deficiency).

6. *Family 76.* Male born 1952. Fig. 7. 3 year old boy, who from the age of 3 months has had several subcutaneous bleedings, intramuscular hematomas and bleedings from the frenulum of the upper lip. He has also had an intestinal hemorrhage. Several cases of hemorrhagic diathesis among male relatives. Laboratory investigations: Clotting time of whole blood (37° C) 60—90 minutes. Quick "prothrombin" 95 per cent. Recalcification time on diluted plasma 10′45″. In the thrombin generation test a very low thrombin concentration was attained (Fig. 7a). An addition of 0.2 ml plasma from a patient with classical hemophilia (AHF deficiency) did not improve the thrombin generation (Fig. 7 b) whereas normal values were obtained on addition of 0.2 ml citrate plasma from a patient with Christmas disease (Fig. 7c).

Diagnosis: Classical hemophilia (AHF deficiency).

DISCUSSION

In 1911 Addis demonstrated by a thrombin generation test that the formation of thrombin in hemophilic blood was delayed. Fischer (1935), Astrup & Fischer (1936) an Astrup (1942) showed that during the clotting of chicken plasma an active substance inducing coagulation was produced, which could be transferred from the plasma undergoing clotting to fresh plasma. They found that the concentration of the clot-promoting factor first increased slowly, but later increased rapidly with timee. After the clot had formed, the concentration of the clot

promoting factor decreased sharply. The present form of the thrombin generation test was developed by Macfarlane & Biggs (1953) and by Pitney & Dacie (1953). These authors emphasized the importance of controlled centrifugation of the blood samples. With increasing speed of centrifugation, the number platelets decreases, whereby the amounts of thrombin formed diminishes. We could confirm these results. Therefore, we were careful to treat all samples in a uniform manner before performing the tests.

The experiments reported here have given results similar to those reported by other investigators, concerning the thrombin formation in normal and hemophilic blood (Macfarlane & Biggs, 1953; Pitney & Dacie, 1953). In addition it is shown that the thrombin generation test is useful in differentiating between the various forms of hemophilia. In fresh plasma samples, with delayed thrombin formation, the clotting defect can be detected by adding different kinds of plasma and sera in small amounts.

During recent years the thromboplastin generation test has been applied in the differential diagnosis between classical hemophilia and Christmas disease (Biggs & Douglas, 1952; Ramot, Angelopoulos & Singer, 1955; Verstraete & Vanderbrouke, 1955; Verstraete, 1955 and Iversen, 1955). This method gives directly an expression of the thromboplastin formation during coagulation and is less time-consuming than the thrombin generation test. On the other hand, it makes use of various plasma, sera and platelet suspensions, whereby a number of uncontrollable and variable factors can be introduced. The thrombin generation test is a simpler and more easily controllable test though more time consuming. Though the results obtained give an indirect expression of the thromboplastic activity they may be more easily interpreted. During the last year we have been working on a classification of hemophilic patients. In this study the thrombin generation test has proven very useful.

Among the first 38 patients studied, 19 patients had classical hemophilia, 12 had Christmas disease, 4 had both Christmas disease and classical hemophilia. One patient had a thromboplastin inhibitor. In 3 patients, hitherto clinically interpreted as hemophiliacs, no clotting defects were detected. The results of these investigations will be presented in detail later.

SUMMARY

The thrombin generation in plasma from hemophilic patients has been studied. The thrombin generation test has been found useful in the differential diagnosis between classical hemophilia and Christmas disease.

ADDITION TO THE PROOF

Detailed investigations of the patients in our "Christmas" group have revealed that this group includes at least two different deficiencies in the thromboplastin stystem. One of these deficiencies is caused by lack of the Christmas facttor. Another is caused by lack of of a new plasma factor, the effect of which can be replaced by a serum factor different from the Christmas factor (Sjølin, in preparation).

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ENZYMIC DETERMINATION OF ADENINE NUCLEOTIDES IN BLOOD

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Biochemical and pharmacological research within the past two decades plainly shows that the adenine nucleotides hold a central position in the basal metabolism of the organism. Until a few years ago quantitative determinations of these substances in blood and tissue were difficult to perform, owing to the fairly low specificity and sensitivity of the earlier measuring methods employed for biological material.

Our search for precursors of the considerable amount of hypoxanthine forming in withdrawn human blood (Jørgensen & Poulsen, 1955) required that we be able to measure the following adenine nucleotides: adenosine-triphosphate (ATP), adenosine-disfofate (ADP) and adenosine-monophosphate (AMP).

Originally, workers utilised the low solubility of certain salts of the nucleotides or the fact that acid hydrolysis causes splitting of the high-energy phosphate bonds in these compounds. These methods being rather inconvenient, only few papers have been published dealing with the adenine nucleotides in man.

Kalckar's enzymatic differential spectrophotometry (1947, a, b, c) represents a great step forward with regard to accuracy, specificity and sensitivity. Enzymatic dephosphorylation of ATP and ADP combined with enzymatic deamination of the resulting AMP renders possible elaboration of a technique particularly suitable for blood, but also useful for all other biological materials.

The method devised by us and presented here does not offer greater difficulties than that for the enzymatic spectrophotometric determination of serum uric acid (Prætorius & Poulsen, 1953). We are therefore of the opinion that adenine nucleotides likewise can be studied with advantage in any clinical laboratory with acces to spectrophotometric equipment fit for measurement within the ultraviolet range.

The determination is made on a trichloracetic acid filtrate. An enzymatically produced change of extinction at 265 mμ allows of a direct simple calculation of the blood concentration of AMP, ADP and ATP.

METHODS

Apparatus:

All optic measurements were carried out in a Beckman's spectrophotometer with ultraviolet attachment. Standard quartz cells $1 \times 1 \times 4$ cm, light path 10 mm were used.

[1] National Science Foundation Postdoctoral Fellow.

Reagents:

Trichloracetic acid 10 per cent (wt./vol.)
N-NaOH
$MgCl_2$ (10 mg/ml)
Glucose 0.5 M (9 g glucose in 100 ml of water).
Malonate buffer 0.6 M, prepared from 6.25 g malonic acid in about 5 ml of water with pH adjusted to 5.9 by adding about 19 ml of N-NaOH. Distilled water quantity sufficient, to make 100 ml.

Substrates:

Adenosine-monophosphate 3.7 mmol/l muscle adenylic, acid, A5MP, AMP). 128.7 mg AMP (Schwarz Lab., N. Y.) was dissolved in 50.0 ml of 0.3 N-HCl. This solutions contains 0.5 mg AMP adenine per millilitre.

Adenosine-diphosphate 3.7 mmol/l (ADP) was prepared from the barium salt $Ba_3(ADP)_2$ (Sigma). 58.5 mg Ba-ADP were dissolved in 3.0 ml of 0.1 N-H_2SO_4 in a graduated flask, and water was thereafter added to 25 ml. The $BaSO_4$ was removed centrifugation. 1.0 ml contains 0.5 mg ADP adenine.

Adenosine-triphosphate 1.85 mmol/l (ATP). 22.4 mg of the sodium salt ATP, Na_2, $3H_2O$ (Sigma) were dissolved in 20.0 ml of distilled water. This solution contains 0.25 mg ATP adenine per ml.

Enzymes:

Adenylic acid deaminase (Schmidt, 1928) was prepared according to Kalckar (1947): About 300 g muscle from a recently killed rabbit is minced and extracted with 3 volumes of water at 4° C for 15 minutes. After filtration through 2 layers of gauze the filtrate is left at 4° C for 18 hours. Owing to acid formation, especially lactic acid, the pH of the extract will fall to about 6. This effects precipitation of the adenylic acid deaminase, as well as other enzymes. The precipitate is isolated and eluated at pH 8 with 1 M ammonium acetate at 4° C while stirring for one hour. The supernatant fluid of the eluate is thereafter fractionated with solid ammonium sulphate, which is added slowly while stirring. The precipitate occurring at between 30 and 50 per cent saturation is suspended in 10 ml of 0.06 M malonate buffer of pH 5.9. Determined photometrically, according to Kalckar (1947 III), this preparation should contain about 2 mg protein per ml.

The activity was as follows: 15 μl of enzyme suspension added to 3.0 ml of adenylic acid solution, about 30 μmol/l in 0.06 M malonate buffer, gave a $-\Delta E_{265}$ mμ of 0.025—0.030 per minute during the half value period.

The inherent extinction of 15 μl of enzyme suspension in 3.0 ml of buffer was 0.008—0,020. being constant for more than 2 hours.

Myokinase was prepared according to Colowick & Kalckar (1943): About 200 g rabbit muscle, excised immediately after the animals have been killed by a blow on the neck, are cooled down to 4° C, minced and extracted twice with 2 volumes of water each time. This extract is acidified with 0.05 volume of N-HCl and heated on a water bath to 90° C. After 2 minutes the solution is rapidly cooled down, and the pH is adjusted to 6.5 with 2 N-NaOH. The precipitate produced is removed by centrifugation. Ammonium sulphate is added to the supernatant fluid to 80 per cent saturation, and the resulting greyish sediment is suspended in 5—10 ml of water. Our preparation contained about 5 mg protein per ml.

Activity: 25 μl of enzyme suspension added to 3.0 ml of ADP solution, about 30 μmol/l in 0.06 M malonate buffer with 15 μl of adenylic acid deaminase, gave a $-\Delta E_{265}$ mμ of 0.010—0.015 per minute during the half value period.

The inherent extinction of 25 μl of enzyme suspension in 3.0 ml of buffer was between 0.060 and 0.085 and was constant for more than 2 hours.

Hexokinase was purchased from the Pabst Laboratories, Milwaukee, Wis. 50 mg of hexokinase-containing powder are dissolved in 1.0 ml of 1 per cent glucose.

Activity: 10 μl of hexokinase solution added to 3.0 ml of ATP solution containing about 30 μmol/l in 0.06 M malonate buffer with 15 μl of adenylic acid deaminase and 25 μl of myokinase, as well as 60 μl of 1 per cent glucose and 60 μl of $MgCl_2$ gave a $-\Delta E_{265}$ mμ of 0.006 per minute during the half value period.

The inherent extinction of 10 μl of hexokinase solution in 3.0 ml of buffer solution was about 0.035 and constant for more than 2 hours.

Storing:

The enzyme preparations were stored in stoppered test tubes at 4 ° C. While being used in connection with Beckman's apparatus these tubes were kept in iced water.

The adenylic acid deaminase and the myokinase preserved their activity unchanged for more than 6 months.

Hexokinase solutions tend to become turbid when more than 4 weeks old. Therefore each time we prepared solutions for only 2—3 weeks' use.

DETERMINATION OF THE ADENINE NUCLEOTIDES IN PURE SOLUTIONS

General remarks. Kalckar indicated the principle for enzymatic spectrophotometric determination of the adenine nucleotides AMP, ADP and ATP. The enzymatic process takes place in the optic cell, under continuous observation in Beckman's apparatus.

The spectra of adenosine, AMP, ADP and ATP are identical with that of adenine. Deamination of the adenine and purine fractions in adenosine and AMP yield, respectively, hypoxanthine and the hypoxanthine compounds corresponding to adenosine and AMP, *i. e.* inosine and inosinic acid (IMP). The spectra of these compounds are identical with that of hypoxanthine, but differ characteristically from the spectra of adenine and the adenine compounds. *The spectra of all these compounds thus depend solely on the purine fraction in the molecule.* An enzymatic hydrolytic deamination of the adenine compound will change its spectrum from an adenine spectrum to a hypoxanthine spectrum. Like Kalckar (1947 b), we compared the two spectra (Fig. 1).

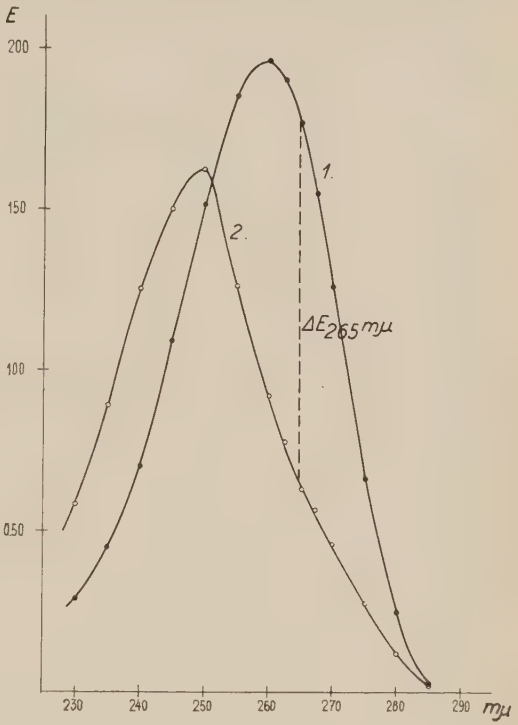

Fig. 1. Absorption spectra of adenylic acid and inosinic acid. Curve 1 is the spectrum before the enzymatic deamination to inosinic acid by adenylic acid deaminase, and curve 2 is the spectrum after the conversion. The adenylic acid concentration is about 20 μmol/l in 0.06 M malonate buffer of pH 5.9.

Abscissae: wave length, mμ.

Ordinates: extinction read against 0.06 M malonate buffer containing the same concentration of enzyme as the reaction mixture.

The two spectra display characteristic differences. The adenine compound shows maximum absorption at 260 mμ and the hypoxanthine compound at 250 mμ. The difference between the two spectra is greatest at 265 mμ ($-\Delta E_{265\,m\mu}$).

If adenylic acid deaminase is added to a solution of all three adenine nucleotides, the

resulting decrease of the extinction of the solution at 265 mμ (— ΔE_{265} mμ) will be due exclusively to the amino group in the AMP being replaced by a hydroxyl group. AMP is thereby converted into IMP, whereas the amino groups i ADP and ATP are left intact. By enzymic determination of ADP and ATP we constantly read — ΔE_{265} mμ, occurring by deamination of AMP, because ADP and ATP are converted step by step into AMP by adding the respective enzymes. Thus, myokinase converts ADP into AMP, and hexokinase dephosphorylates ATP forming ADP. If myokinase is added to a solution of the three adenine nucleotides, after all AMP has been deaminated by adenylic acid deaminase, the resulting — ΔE_{265} mμ will be due exclusively to the fact that the AMP produced by ADP is converted into IMP. Finally, addition of hexokinase, which converts ATP into ADP will produce a third — ΔE_{265} mμ because the ADP will be dephosphorylated and deaminated by the two enzymes already present.

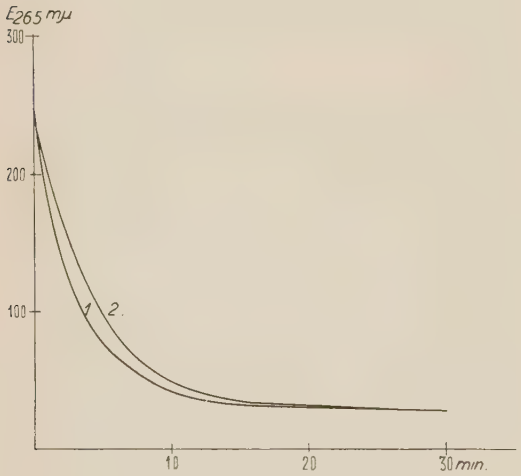

Fig. 2. The enzymatic deamination of adenylic acid (AMP) by adenylic acid deaminase at pH 5.9. The initial adenylic acid concentration about 30 μmol/l in 0.06 M malonate buffer without (curve 1) and with (curve 2) 1 per cent neutralized trichloracetic acid.

Abscissae: reaction time, minutes.

Ordinates: E_{265} mμ.

At zero time 15 μl of adenylic acid deaminase are added to 3.0 ml of solution.

We can thus step by step determine the concentration of each component of a solution containing all three adenine nucleotides.

Determination of AMP. Measured at 265 mμ, addition of adenylic acid deaminase to a solution of AMP produced an extinction decrease. Fig. 2, curve 1, shows that the extinction decreased at a fairly constant rate the first few minutes only. The decrease ceased 20 minutes after the addition of the enzyme. The presence of 1 per cent neutralized tricloracetic acid reduced the rate of the process, but the total amount of AMP was nervertheless converted within 20 minutes. This appears from the results of recovery experiments and experiments showing proportionality between the substrate concentration and the enzymatically produced extinction decrease .

Table I shows proportionality between the concentration of AMP and the extinction decrease — ΔE_{265} mμ.

Determination of ADP. Fig. 3, curve 1, shows — ΔE_{265} mμ following the dephosphorylation produced by myokinase and succeeding deamination of ADP in a solution already containing adenylic acid deaminase. During the first few minutes after addition of myokinase the extinction decrease proceeded at a constant rate. The decrease ceased after 20 to 30 minutes.

Table I. *Proportionality is seen between the concentration of adenylic acid (AMP) and* $-\Delta E_{265}$ *mμ in the enzymatic deamination of AMP by adenylic acid deaminase. Reaction time 30 minutes. The substrate was dissolved in 0.06 M malonate buffer of pH 5.9.*

Concentration of AMP	μg/ml as adenine	2	4	6	8	10
	micromoles per litre	14⅔	29⅓	44	58⅔	73⅓
$-\Delta E_{265}$ mμ	in malonate buffer pH 5.9	0.110	0.215	0.333	0.439	0.550
	in malonate buffer pH 5.9 with 1 per cent neutralized trichloracetic acid	0.111	0.219	0.336	0.442	0.559

1 per cent trichloracetic acid inhibited this reaction, too, but a constant extinction was here obtained within 30 minutes.

Table 2 shows proportionality between the ADP concentration and $-\Delta E_{265}$ mμ.

By comparing $-\Delta E_{265}$ mμ in Tables I and II it is seen that the enzymatic deamination of ADP with myokinase and adenylic acid deaminase produced an extinction decrease that was only half of the $-\Delta E$ obtained by deamination of an equimolar amount of AMP. This is due to the action of myokinase on ADP. Myokinase converts two molecules of ADP into one molecule of ATP and one of AMP (Kalckar, 1943). The observed extinction decrease, and thus the amount of deaminated AMP accordingly represents no more than half of the ADP present at the beginning of the process.

Determination of ATP. Addition of hexokinase, myokinase and adenylic acid deaminase to a solution of ATP brought about a $-\Delta E_{265}$ mμ corresponding to the equimolar amount of AMP produced by dephosphorylation. After 1¾ to 2 hours the extinction decrease had practically ceased; however, beyond 2 hours a very slow fall could, occasionally be seen, which was independent of the amount of ATP originally present. The difference between the enzymatically produced extinction decrease and that irrelevant to the measurement is,

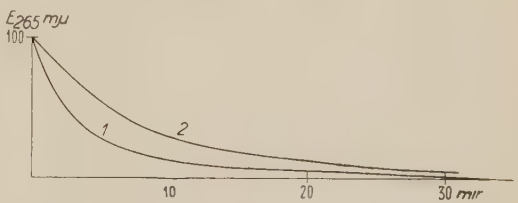

Fig. 3. The enzymatic dephosphorylation and deamination of adenosine-disphosphate (ADP) by myokinase and adenylic acid deaminase respectively at pH 5.9. The adenosine-diphosphate concentration about 30 μmol/l in 0.06 M malonate buffer without (curve 1) and with (curve 2) 1 per cent neutralized trichloracetic acid.

Abscissae: reaction time, minutes.

Ordinates: E_{265} mμ.

At zero time 25 μl of myokinase and 15 μl of adenylic acid deaminase are added to 3.0 ml of solution.

Table II. *Proportionality is seen between the concentration of adenosine-diphosphate (ADP) and $-\Delta E_{265}$ mμ on enzymatic dephosphorylation and demination of ADP by myokinase and adenylic acid deaminase. Reaction time 30 minutes. The substrate was dissolved in 0.06 m malonate buffer of pH. 5.9.*

Concentration of ADP	μg/ml as adenine	2	4	6	8	10
	micromoles per litre	14⅔	29⅓	44	58⅔	73⅓
$-\Delta E_{265}$ mμ	in malonate buffer pH 5.9	0.055	0.110	0.162	0.216	0.273
	in malonate buffer pH 5.9 with 1 per cent neutralized trichloracetic acid	0.055	0.106	0.155	0.214	0.245

so great that reading of the former offers no difficulty in practice.

Table III shows the relation between the ATP concentration and $-\Delta E_{265}$ mμ within an observation time of 2 hours. Presence of ½ per cent trichloracetic acid gave the same $-\Delta E$. 1 per cent trichloracetic acid, on the other hand, caused the reaction to proceed at a very slow rate, so that the process was not completed within a reasonable time. Since the activities of adenylic acid deaminase and myokinase are not influenced by this trichloracetic acid concentration, it must be the activity of the hexokinase solution used that was so greatly reduced.

We tried to raise the rate of the extinction decrease by letting the reaction with all three enzymes take place at 37° C. However, in a solution containing trichloracetic acid the reaction was greatly inhibited at this temperature. This necessitad changing to a temperature of 20° C (room temperature).

We can calculate from Tables I—III that the enzymatic deamination of 1 mmol/l of AMP and ATP produces a $-\Delta E_{265}$ mμ of 7.50, which means that

1 mmol/l, 1 cm
K_{265} mμ for AMP and ATP is 7.50 while
1 mmol/l, 1 cm.
K_{265} mμ for ADP is 3.75.

K is the change of extinction taking place by deamination in a solution containing 1 mmol/l of the adenine nucleotide concerned.

Determination of adenine nucleotides in blood.

2.00 ml of heparinized blood are injected in a strong, but thin jet into 2.00 ml of 10 per cent trichloracetic acid in a centrifuge tube to obtain a uniform deproteinization. After centrifugation, 2.00 ml of the supernatant fluid are transferred to a 10 ml graduated flask and neutralized with 200 μl of N-NaOH, and the pH is fixed at 5.9 by adding 1 ml of 0.6 M malonate buffer. Then, 200 μl of glucose and 200 μl of $MgCl_2$ are added, and finally, distilled water, quantity sufficient to make 10 ml.

Table III. *Proportionality is seen between the concentration of adenosine-triphosphate (ATP) and $-\Delta E_{265}$ mμ on enzymatic dephosphorylation and deamination of ATP by hexokinase, myokinase and adenylic acid deaminase. Reaction time 2 hours. The substrate was dissolved in 0.06 M malonate buffer of pH 5.9.*

Concentration of ATP	μg/ml as adenine	2	4	6	8
	micromoles per litre	14⅔	29⅓	44	58⅔
$-\Delta E_{265}$ mμ	in malonate buffer pH 5.9	0.091	0.209	0.326	0.449
	in malonate buffer pH 5.9 with ½ per cent neutralized trichloracetic acid	0.106	0.222	0.332	0.440

3 ml of this solution are transferred to a quartz cuvette for determination of the AMP and ADP concentration. The reference cuvette contains the same solution. The optic density of the reference cuvette with its contents is defined as an extinction reading at 0.100, 0.200 or 0.300 at 265 mμ, according to the extinction decrease expected by adding first adenylic acid deaminase and thereafter myokinase to the measuring cuvette. This corresponds in practice to placing the galvanometer needle at zero at change of slit width and sensitivity, and adjusting the transmission scale to either 0.100, 0.200 or 0.300 when the reference cuvette is translumination.

15 μl of adenylic acid deaminase are added (zero time 1), and by repeated measurements of extinction at 265 mμ within the first minute, extrapolation can be performed to the extinction at zero time. The final extinction is read after 30 minutes. The total $-\Delta E_{265}$ mμ divided by 0.0075 gives the AMP concentration of the cuvette contents in μmol/l.

At this stage (zero time 2) 25 μl of myokinase are added to the same cuvette. Renewed extrapolation gives the extinction at zero time. The final extinction is read 30 minutes later. $-\Delta E_{265}$ mμ divided by 0.0037 gives the ADP concentration of the cuvette contents in μmol/l.

The total concentration of adenine nucleotide (AMP + ADP + ATP) is determined in a portion of the solution in the graduated flask. This solution is diluted with an aliquot of water, and 3 ml are transferred to a quartz cuvette. Dilution is necessary, because a trichloracetic acid concentration exceeding ½ per cent causes too great an inhibition of the hexokinase preparation. A cuvette with the same contents is used as reference. Similarly, the transmission scale is adjusted to 0.100, 0.200 or 0.300, dependent on the expected extinction decrease, at translumination of the reference cuvette and variation of slit width or sensitivity. Then follows addition of 20 μl of hexokinase, 25 μl of myokinase and finally, 15 μl of adenylic acid deaminase, and the extinction

Table IV. *Recovery of AMP, ADP and ATP added to a suspension of blood cells in Tyrode-Locke's solution.*

Added nucleotide	Micromoles per litre	Per cent recovered as AMP	ADP	Total recovery in per cent
AMP	75	90		90
	300	96		96
ADP	150	63	29	92
	600	32	43	75
AMP+ADP	75+150	72	34	106
AMP+ADP	300+300	62	26	88
ATP	150			93
	300			87
	450			90

at zero time is determined. The final extinction is read 2 hours later. $-\Delta E_{265}$ mμ divided by 0.0075 gives the total concentration of adenine nucleotide in the cuvette contents. As we know the sum of the concentrations of AMP and ADP, and now also the concentration of total adenine nucleotide in blood, the ATP concentration is found as the difference between these values.

Recovery of adenine nucleotides added to blood.

After centrifuging 5 ml of freshly withdrawn heparinized blood 2.00 ml of plasma are replaced by Tyrode-Locke's solution (Ahlgreen, 1930) containing known amounts of AMP, ADP or ATP.

Table IV shows that more than 90 per cent of an added amount of AMP was recovered. Added ADP was recovered partly as such and partly as AMP, *i. e.*, dephosphorylation had taken place from the moment plasma had been replaced by ADP-containing Tyrode's solution until deproteinization occurred, a period of a few minutes.

The recovery of ATP was determined by measuring the total nucleotide concentration in blood with and without ATP added.

The loss in these recovery experiments may be due to the deproteinization or to the fact that the adenine nucleotide was broken down to other compounds during the interval from the addition to the deproteinization.

DISCUSSION

The technique here described offers the advantage of allowing measurement of all three adenine nucleotides in the same filtrate and at the same pH. The specificity of the method has been insured partly by measuring at a definite wave length within the ultraviolet range and partly by the specificity of the adenylic acid deaminase. It is important that the adenylic acid deaminase does not posess myokinase activity, for if so, ADP, if present in the measuring solution, will be broken down together with AMP and be measured as such. Contrariwise admixture of adenylic acid deaminase to the myokinase used presents no problem, because when we

measure ADP the myokinase is added to a solution already containing adenylic acid deaminase and consequently no AMP.

The preparation of adenylic acid deaminase and myokinase may sound rather laborious, but it is a grateful task. Each time we produced the enzymes we obtained preparations with excellent activity. Our myokinase showed little adenylic acid deaminase activity, while, on the other hand, our adenylic acid deaminase by no means broke down ADP.

The purchased hexokinase is a freeze-dried powder prepared by alcohol precipitation of an extract of autolysed yeast. It probably contains many other enzymes. A content of enzymes that dephosphorylate ADP and AMP, thereby producing adenosine, is a conceivable source of error. Such adenosine would not be deaminated by the adenylic acid deaminase. The results of our experiments with pure ATP solutions and of our recovery experiments proved, however, that no such enzymes are contained in the powder.

Albaum & Lipschitz (1950) likewise measure all three adenine nucleotides in blood spectrophotometrically by deamination of AMP, but in a sligthly different way. They utilise the rate of $-\Delta E_{265}$ mμ the first 10 minutes after addition of adenylic acid deaminase, having found proportionality between the AMP concentration and this extinction decrease. ADP and ATP are determined by measuring the AMP concentration in portions of the same solution, where ADP and ATP have first been converted to AMP by adding myokinase alone and myokinase plus hexokinase respectively. The difference between the AMP concentrations of these three solutions is taken to indicate the concentrations of ADP and ATP. The concentrations of the adenine nucleotides are read from standard curves plotted by means of known solutions with the same concentration of the deproteinizing agent (2 per cent perchloric acid) as the unknown filtrate.

The concentration of the deproteinizing agent in the filtrate varies, of course, with the protein concentration in the original sample, and the activity of the enzymes used is inhibited, dependent on the perchloric acid concentration. We therefore chose to employ the changes of extinction resulting from total deamination of the adenine nucleotides as an expression of their concentrations.

Kornberg (1950) has indicated a method for isolated enzymic spectrophotometric determination of ATP: When ATP is converted into ADP with hexokinase, the glukose-6-phosphate produced is converted into 6-phosphogluconic acid, while at the same time added diphosphopyridine nucleotide (DPN) is converted into $DPNH_2$. This latter reaction is catalysed by glucose-6-phosphate dehydrogenase (Zwischenferment) and measured by the extinction increase at 340 mμ.

Several workers proceed by measuring the adenine nucleotide concentration by enzymatic dephosphorylation and determination of the thereby liberated phosphate (Bailey 1949; Mackler *et al.*, 1954). The method indicated by some Scandinavian writers (Rehell *et al.*, 1952) for measuring ATP in blood is based on the same principle. An enzyme, possibly potato apyrase, prepared from potates (Lee & Eiler, 1951) splits off the terminal phosphate from ATP. However, at temperatures above 17° C the terminal phosphate will also be liberated from the ADP produced or originally present, so

the reaction cannot be said to be absolutely specific.

Provided one has acces to a spectrophotometer with ultraviolet equipment, the technique indicated by us seems to offer considerable advantages. A trained analyst using this technique can perform 15 determinations of all three adenine nucleotides in about $2^1/_2$—3 hours.

SUMMARY

A method for determining the adenine nucletotides ATP, ADP and AMP in human blood is described in detail.

For the analysis 2.00 ml of heparinized blood is deproteinized in 2.00 ml of 10 per cent trichloracetic acid. The supernatant fluid is buffered at pH 5.9 with malonate buffer. Dephosphorylation of ATP and ADP with hexokinase and myokinase is combined with deaminatioin of AMP with adenylic acid deaminase. This latter reaction is measured spectrophotometrically at 265 mμ. There is proportionality between the concentration of each adenine nucleotide and this extinction decrease.

Experiments with each of the adenine nucleotides or combinations of these added to blood in concentrations of 75 μmol/l to 450 μmol/l (about 2.5 mg/100 ml to about 23 mg/100 ml) showed recovery of more than 90 per cent.

ACKNOWLEDGMENTS

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A CORRECTION FACTOR FOR HELIUM ABSORPTION IN LUNG VOLUME DETERMINATIONS

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In the method for the determination of the lungs' functional residual capacity (FRC) using a helium-air gas mixture in a closed system, Meneely & Kaltreider (1949) recommend the subtraction of 110 ml from the determined value as a correction for absorbed helium. No data were presented to support the derivation of this correction and the authors were not convinced that is was correct. Holmgren (1954), on the other hand, introduced no correction for absorbed helium because he felt that the catharometer's sensitivity was to low to detect the small quantity of helium which would be absorbed. He calculated the latter to be between 4 and 21 ml in 5 minutes.

The problem has been taken up anew since the methodologic error caused by helium absorption may be of significant size and not particularly variable from patient to patient. A specific investigation as to the magnitude of this absorption was therefore performed.

METHOD

The arrangement of the apparatus was similar to that described by Holmgren (1954). An ordinary spirometer of Knipping type was used. A CO_2 absorber was present between the bell and the fan; the latter moved the gas present in the system at a flow rate of about 50 liters per minute. Connected parallel to the fan was a Cambridge catharometer graduated for concentrations of helium of 0—20 per cent in air. The flow of gas through the catharometer was controlled with a flow meter and valve to about 200 ml per minute. The volume of the apparatus exclusive of the spirometer bell was 3.1 liters.

Observation of CO_2 absorption in 15 studies showed no significant difference between the last reading of the procedure and one made one minute after the patient was disconnected from the spirometer system. Therefore, since fresh sodium carbonate was used, CO_2 absorption was assumed to be complete.

Leakage in the system was investigated during 5 studies lasting 10 minutes each, and in 5 others lasting 20 minutes each. Readings were made every minute for the first 10 minutes and then every fifth minute immediately after the current to the catharometer was checked. Leakage was never seen to occur. Statistical treatment of the 100 readings showed a standard deviation of 0.02 per cent. Since the O_2 concentration in the system was held constant by a continuous O_2 supply, no error in the helium concentration could have occurred from this cause.

Before each investigation a mixture of about 18 per cent helium in air was introduced into the spirometer. After the subject was connected to the system, the helium concentration fell gradually to about half the initial value. The total quantity of gas in the system was kept at about 4.5 liters (varying between 4.3 and 4.8 liters) with a continuous oxygen supply. The volume of the system plus

[1] With the technical aid of A. Åhbeck.

the patient varied on an average ± 0.03 liter during any given procedure.

Two healthy individuals (laboratory personnel) with unusually stable pulmonary end-expiratory levels were chosen as experimental subjects. One (E. S.) was a man 188 cm tall weighing 82 kg. The other (B. O.) was a woman 169 cm. tall weighing 53 kg. When investigations were repeated, at least a 15 minute period intervened. Each investigation lasted 10 minutes and readings were taken every minute. In order to obtain good mixing as quickly as possible, five deep respirations were performed at the beginning of each study. The time lag of the catharometer was about one minute so that each reading was related to the volume registered one minute before. The accuracy of the readings of helium concentration was 0.05 per cent which means that with a volume of about seven liters in the system, a difference of 3—4 ml of helium could be detected.

In all the investigations the mixing was complete after three minutes. After this time only small changes in helium concentration took place, and these could be attributed to absorption. The FRC was calculated after each investigation using the three minute reading for helium concentration and spirometer volume. The quantity of helium present in the system was then determined for each minute of the study using the corresponding spirometer volume, the reading of the helium concentration, and the FRC which was assumed to be constant during the study.

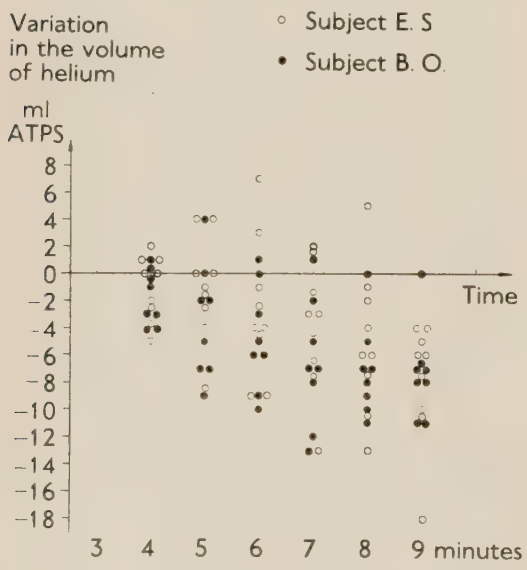

Fig. 1. Changes in the volume of helium taking place in 17 studies on 2 normal subjects 3 minutes after the subject was connected to the spirometer.

RESULTS

The observations refer to the two experimental subjects who together performed 17 tests (9 for one, 8 for the other). Figure 1 is a diagram of the results. A decrease in the quantity of helium present took place in each instance although the limited accuracy of the helium concentration measurements caused a large spread of the collected values. However there was a definite tendency toward a steady decrease in the quantity of helium in the system. The helium loss from the third to the sixth minute was 3.65 ml ± 1.06, or approximately 4 ml. The loss from the third to the ninth minute was 7.65 ml ± 0.94, or approximately 8 ml. The differences between the third minute value, the sixth minute value and the ninth minute value were statistically significant. If a mean loss of 4 ml takes place between the third and sixth minutes, an equal or greater loss could be expected during the first 3 minutes when the subject is unsaturated. Thus during the first 6 minutes, at least 8 ml should be lost due to absorption, and after 9 minutes, 12 ml. Eight ml of helium under the conditions of the study corresponds to a lung volume of 8/700 of 7,500 ml, *i. e.* 86 ml ATPS or 95 ml BTPS. This then should be the amount subtracted from the 6 minute volume value obtained in order to eliminate the effect of helium absorption. Similarly, if a 7 minute

tudy were performed, the reduction would e 105 ml BTPS; for a 9 minute procedure, 27 ml BTPS.

DISCUSSION

A theoretical calculation of the absorption f helium can not be taken up without know-edge of helium's rate of diffusion into the ody tissues. However if this is assumed to e similar to that of hydrogen (with which elium shares many properties), one can nake an approximate calculation. Since ydrogen's solubility coefficient is almost wice that of helium (Kety, 1951) and since he accuracy of the combustion method for he determination of hydrogen is greater han the catharometer method for helium determination can be made on 2—3 ml of ydrogen after dilution up to 7 liters), it is ossible to determine the absorption rate of ydrogen with fair accuracy. The method equires two or more serial measurements f hydrogen concentration in a closed respira-ory system after complete mixture of the as contents of the lungs and the spirometer as taken place.

Such a determination was performed by ne of us (Birath, 1944) in 27 subjects with nixing tested every minute. Complete mixing vas seen after 2 minutes; this corresponds vell to the theoretical calculation that 25—30 reaths would result in 99 per cent mixing. Hydrogen determinations were made every ninute between the third and ninth minutes. During these 6 minutes the lung volume had pparently increased 0.19 liter BTPS $\pm$ 0.01 vhich was attributed to hydrogen absorption. The mean difference between the third and ixth minutes was 0.092 liter. Calculated er minute, the mean absorption was O.031 liter. Incomplete mixing could not have accounted for this.

If the rate of diffusion for helium in the body tissues were the same as for hydrogen, and the solubility about half $\left(\frac{0.0097}{0.018}\right)$ a theoretical correction of about 0.017 liter BTPS per minute could be used. For a seven minute procedure, 119 ml BTPS would be subtracted, closely resembling the value used by Meneely & Kaltreider (1949) of 110 ml.

The mean value of 105 ml at the seventh minute derived from the present study (17 investigations in two normal subjects) is thus in fairly good agreement with the suggested value of 110 ml and theoretically derived value of 119 ml. Although the absorption of helium in different individuals and under different experimental conditions is dissimilar some correction should be made. Otherwise the derived values for lung volumes and their subdivisions will include an eliminatable methodologic error.

SUMMARY

A theoretical calculation of the absorption of helium during the measurement of lung volumes in a closed respiratory system based upon a previous study of hydrogen absorption under similar circumstances leads to the conclusion that the lung volume value derived from a seven minute procedure ought to be corrected by subtraction of 119 ml BTPS.

Direct determinations of helium absorption under similar circumstances leads to capacity estimations with measurements of the quantity of helium present in the system every minute after an initial complete

mixing had taken place. The mean value of 17 such studies for the seventh minute in two normal subjects corresponds to 105 ml BTPS of lung volume, and the use of this figure as a correction to diminish lung volumes obtained with a seven minute closed system helium dilution technique is recommended.

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HEMOGLOBIN IN PERNICIOUS AND ALLIED ANEMIAS THE SIGNIFICANCE OF AN ABNORMAL ALKALI DENATURATION CURVE

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Alkali hematin is formed when alkali is ded to hemoglobin solutions. The dena- ration rate is slower for fetal hemoglobin an for normal adult hemoglobin, and the action has therefore been used to distin- ish between these two types of hemoglobin. he purpose of this paper is to show that ood from patients suffering from pernicious d allied anemias yields abnormal alkali- naturation curves, but these curves do not cessarily indicate the presence of abnormal moglobin.

INTRODUCTION

According to Krüger & Gerlach (1926) e presence of a pathological hemoglobin in ses of pernicious anemia was postulated dependently in 1925 by Bischoff, Wörpel, d Schultze who all used the alkali denatura- on method. In 1930 Schenk reported emical abnormalities in pernicious anemia moglobin. In 1934 Barcroft found the xygen dissociation curve of hemoglobin in ses of anemia to resemble that of fetal moglobin. Barcroft further pointed out at in some species of animals the presence fetal hemoglobin was associated with an creased erythrocyte diameter.

One of us (G. L.,1949) has shown that the increased erythrocyte diameter as revealed by an abnormal Price Jones curve, seen in pernicious anemia, is due to the presence of macrocytes. Macrocytes may be demonstrated by measuring red cells in a blood smear, and form a distinct class of cells with diameter 0.75—1.00 micron above normal values. The dried macrocyte is thinner than the dried mormocyte (averaging 0.38 micron against normal 0.60 micron). In the dried blood smear the volume of the macrocyte averages 23 cu.micron against normal 27 cu.micron.

In the wet state the presence of macrocytes can not be demonstrated: Samples of blood containing macrocytes have a mean corpuscular volume within normal limits, and diameter measurements made on blood cells suspended in plasma and placed on a non-wettable surface yield nearly normal Price Jones curves. (Larsen, 1952, 1955).

These findings strongly suggest a difference in physical behaviour: The macrocytes flatten out to a greater degree, and shrink more during the act of drying, than do normal cells when a blood film is made. It seemed reasonable to examine the possibility that these differences in physical behavior were due to the presence of an

[1] This investigation was aided by a grant from ordisk Insulinfond.

abnormal hemoglobin in the macrocytes. For it is known that fetal and allied hemoglobins differ from normal adult hemoglobin in such physical properties as solubility, monomolecular layer formation, crystallization, and electrophoretic mobility (Kendrew, 1949).

Using the method to be described below, we constantly found abnormal (kinked) alkali denaturation curves in cases of pernicious and related anemias with macrocytosis. When, following treatment, the macrocytosis disappeared, the alkali denaturation curve became normal (Larsen, 1950). In 1950 the method of alkali denaturation was the only method available to us for demonstrating abnormal hemoglobins. Since then a considerable number of methods has been applied for identification of abnormal hemoglobin and we have used a number of these methods in order to identify the supposed abnormal hemoglobin in pernicious anemia further, but without success:

Hemoglobin from cases of pernicious anemia *crystallized* like normal adult hemoglobin, as found previously by Jope & O'Brien (1949). Dr. E. R. Holiday very kindly examined some of our samples with his *spectrographic method*, but was unable to demonstrate the presence of fetal hemoglobin. We did not find any irregularities by the *salting out method* of Roche & Derrien (1953), and *paper electrophoresis* by the method of Spaet (1953) did not confirm our findings. Normal electrophoresis of pernicious anemia hemoglobin has also been reported by Waris (1954). We have tried to demonstrate *specific precipitins* by the method of Darrow, Nowakovsky & Austin (1940) and to distinguish the supposed different hemoglobins by the *method of frothing* (Dognon & Gougerot, 1948) without success.

We have therefore been forced to reconsider the significance of the abnormal alkali denaturation curves we so constantly observed in cases of pernicious and related anemias.

METHODS

Alkali denaturation was studied by a method based on the method of Brinkman, Wildschut & Wittermans (1934).

10 ml venous blood, with heparin as anticoagulant, was centrifuged and the red cells were washed twice in saline. The red cells were suspended in a phosphate buffer solution (5.18 g $Na_2HPO_4 \cdot 12\,H_2O$ to 1000 ml) and 2 drops of a freshly prepared 0.5 per cent solution of saponin were added to ensure complete hemolysis. The phosphate buffer solution had an osmolarity equivalent to 0.34 per cent NaCl solution and allowed for segregation of resistant blood cells (not dicussed in this paper). The pH of the solution at 20° was 8.75. The optical properties of hemoglobin solutions so prepared did not differ from hemoglobin solutions at pH 7.4. The addition of heparin and saponin did not influence the results.

10 ml of the clear hemoglobin solution, suitably diluted with phosphate buffer solution to optical transmission 40 per cent, was placed in a Lumetron photometer with filter 580. $\frac{N}{1}$ NaOH, 0.35 ml was added and raised the pH from 8.75 to 12.34. The optical transmission per cent was read every 15 seconds for 5 minutes. The sample was left overnight and a final reading was taken, as it was assumed that all hemoglobin then was changed to alkali hematin. The whole procedure was carried out at a room temperature of 20° ± 0.5°.

Interpretation. Curve analysis.

The observed values of optical density (2 — log transmission per cent) were plotted against time in a diagram (Fig. 1). The optical denstity value at zero time was also marked out in the right border of the diagram, and the optical density

value read off after 24 hours was marked out in the left border. A line was drawn between these two points and by means of the per cent scale on top of the diagram the percentage of unaltered hemoglobin present in the solution at each stage of the experiment was estimated.

The log per cent values for unaltered hemoglobin were then plotted against time (Fig. 1, lower half). Where normal adult blood and fetal blood (cord blood) were concerned, these values were grouped around straight lines, but the slopes of these lines were widely different, indicating that the reaction proceeded much more slowly where fetal blood was concerned.

In cases of pernicious anemia, however, the values for log per cent unaltered hemoglobin did not fall on a straight line (Fig. 1, right). The curve was kinked, the first part of the curve having a slope similar to that of normal blood, while the latter part of the curve had a slope similar to that of cord blood. This is generally held to indicate that the sample contains a mixture of normal adult hemoglobin and of a pathological hemoglobin resembling fetal hemoglobin (Jonxis, 1949).

The amount of "fetal hemoglobin" was estimated by extrapolating the log per cent unaltered "fetal hemoglobin" from the lower right hand diagram in Fig. 1. The anti-log values gave the supposed amount of "fetal hemoglobin" at each stage of the experiment, and by simple calculation the amount of "adult normal hemoglobin" was estimated. Finally, the log per cent values of supposed "unaltered adult hemoglobin" was plotted in the diagram, and when these values fell around a straight line with a slope similar to that of adult normal blood, it was taken as proof that the curve analysis was correct.

EXPERIMENTS

Experiment 1 (Fig. 2).

Fig. 2 shows the log per cent values for unaltered hemoglobin in samples from 8 normal adults, 5 samples of cord blood and in 10 samples from patients with pernicious anemia. Cord blood and normalt adult blood invariably yield straight linear curves with a slope characteristic of each type of blood. Patients with pernicious anemia yield kinked curves. In all we have examined 94 samples from 43 cases of pernicious and allied anemias. In 32 patients, with in all 69 observations, a kinked curve was obtained. In all these patients the phenomenon was reproducible, in some cases even after normal values for Hb and R.B.C. were obtained after treatment. But when macrocytosis, which was invariably present in these cases, disappeared, the curves became straight like those seen in normal subjects. The phenomenon was most pronounced in cases with macrocytic anemia of sprue and liver diseases.

In 16 cases of pure pernicious anemia, all in partial remission, and all with only slight macrocytosis, the curves for log per cent unaltered hemoglobin resembled those obtained from normal adult blood.

Experiment 2 (Fig. 3).

The kinked curve for log per cent unaltered hemoglobin is commonly held to indicate the presence of two types of hemoglobin with different reaction time to alkali.

If this is so, addition of normal adult hemoglobin to the sample should shift the kink of the curve towards the right. Fig. 3 shows this expected shift when cord blood is mixed with 25 per cent, 50 per cent and 75 per cent normal adult blood. The values in brackets give the amount of fetal hemoglobin calculated from the curves, and these values correspond fairly well with the amount of cord blood known to be present in each sample. It will be seen that all calculated values are slightly inferior to the true values. This is due to the initial deflection of the curve during the first 15 seconds of the

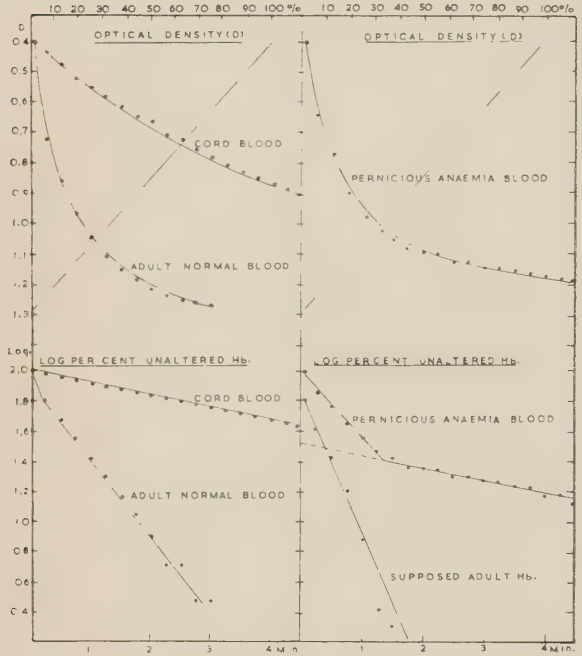

Fig. 1.

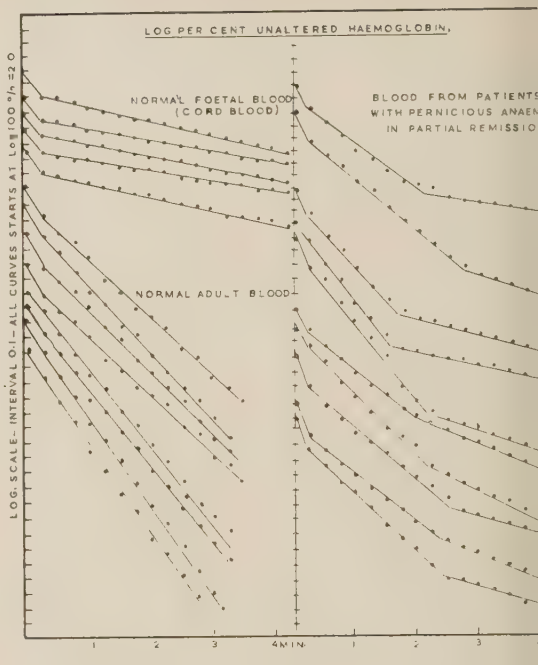
LOG PER CENT UNALTERED HAEMOGLOBIN.
NORMAL FOETAL BLOOD (CORD BLOOD)
BLOOD FROM PATIENTS WITH PERNICIOUS ANAEMIA IN PARTIAL REMISSION
NORMAL ADULT BLOOD
LOG SCALE - INTERVAL 0.1 - ALL CURVES STARTS AT LOG 100 % = 2.0
4 MIN.
4 MIN.

Fig. 2.

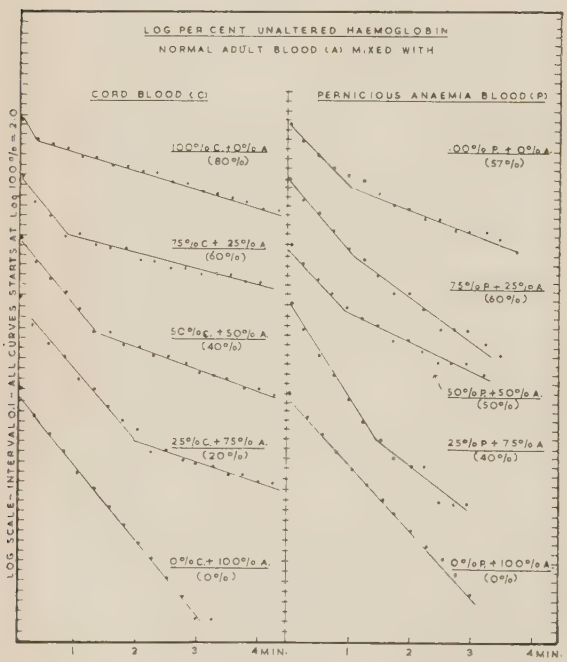
LOG PER CENT UNALTERED HAEMOGLOBIN
NORMAL ADULT BLOOD (A) MIXED WITH
CORD BLOOD (C)
PERNICIOUS ANAEMIA BLOOD (P)
100 % C + 0 % A (80 %)
75 % C + 25 % A (60 %)
50 % C + 50 % A (40 %)
25 % C + 75 % A (20 %)
0 % C + 100 % A (0 %)
100 % P + 0 % A (57 %)
75 % P + 25 % A (60 %)
50 % P + 50 % A (50 %)
25 % P + 75 % A (40 %)
0 % P + 100 % A (0 %)
LOG SCALE - INTERVAL 0.1 - ALL CURVES STARTS AT LOG 100 % = 2.0
4 MIN.
4 MIN.

Fig. 3.

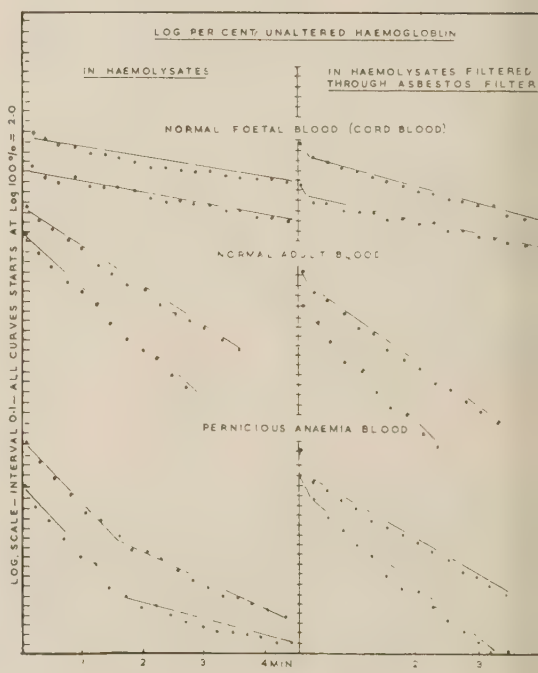
LOG PER CENT UNALTERED HAEMOGLOBIN
IN HAEMOLYSATES
IN HAEMOLYSATES FILTERED THROUGH ASBESTOS FILTER
NORMAL FOETAL BLOOD (CORD BLOOD)
NORMAL ADULT BLOOD
PERNICIOUS ANAEMIA BLOOD
LOG SCALE - INTERVAL 0.1 - ALL CURVES STARTS AT LOG 100 % = 2.0
4 MIN.

Fig. 4.

xperiment. This inflection is probably due o air bubbles introduced with the addition f NaOH.

When the same experiment is carried out ith pernicious anemia blood, no shift of he kink of the curve to the right is observed. he amount of supposed pathological hemo- lobin calculated from the curves varies round 40—60 per cent irrespective of the mount of normal blood which has been dded to the sample.

Experiment 3 (Fig. 4).

All our results so far have been obtained n solutions of hemolysed red cells, no parti- ular care has been taken to ensure pure emoglobin solutions. In Fig. 4, the log per ent values for unaltered hemoglobin, btained by our usual method, are compared ith similar curves obtained by examining emoglobin solutions which have been fil- tered four times through 50 per cent asbestos filter. The filtering does not influence the slope of the curves where cord blood or normal adult blood are concerned. But when hemoglobin solutions from patients with pernicious anemia are filtered, the kinked curves becomes straight, with a slope similar to that of normal adult hemoglobin.

Experiment 2 makes it improbable that hemoglobin itself is the cause of the abnormal curves found in cases of pernicious anemia, and experiment 3 suggests that an active substance is removed by filtration.

Experiment 4 (Fig. 5).

Acting on a suggestion made by G. H. Beaven the following experiment was carried out:

50 ml heparinized blood from newborn (cord blood), from normal adults, and from patients with pernicious anemia were centrifuged. The plasma and the leukocyte layer were carefully removed separately, and the red cells were hemolysed by addition of 300 ml distilled water. After renewed centrifugation the hemolysate was removed and the remaining stroma washed with saline and resuspended in 4 ml saline.

Samples of full blood from normal adults and from patients with pernicious anemia, 10 ml each, were incubated at 37° for 8 hours with plasma, leukocyte suspensions, and stroma suspensions from different sources. The denaturation rates of these samples were then examined and compared with corresponding samples prepared in the usual way.

Incubation with plasma or leukocyte suspensions from different sources did not influence the slope of the curves. Only

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ig. 1. Optical density curve, and curve for log er cent unaltered hemoglobin. Cord blood, normal dult blood and blood from a case of pernicious anemia.

ig. 2. Curves for log per cent unaltered hemo- lobin of fetal (cord) blood, normal adult blood, nd blood from patients with pernicious anemia.

ig. 3. Curves for log per cent unaltered hemo- lobin of fetal (cord) blood and blood from a atient with pernicious anemia, when the samples re diluted with normal adult blood. Numbers in rackets give the amount of pathological hemo- globin estimated by analysis of the curves.

ig. 4. Curves for log per cent unaltered hemo- lobin of fetal (cord) blood, normal adult blood and ernicious anemia blood before and after purifying the hemoglobin solutions by filtering through asbetos filter (50 per cent).

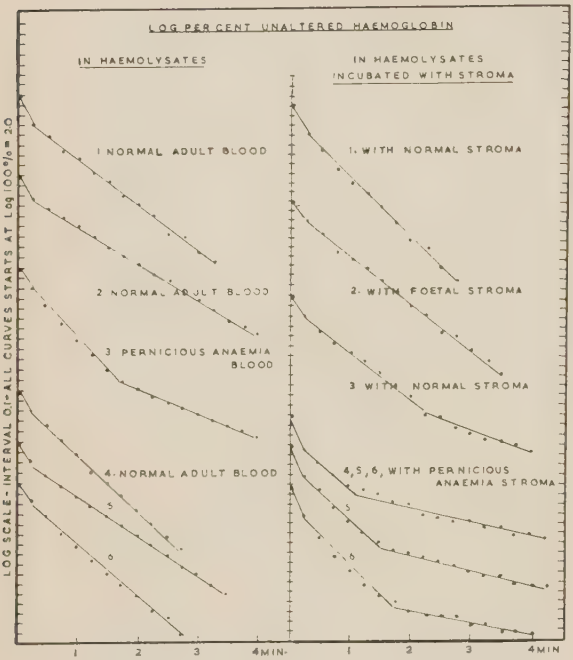

Fig. 5. Curves for log per cent unaltered hemoglobin of normal adult blood and pernicious anemia blood before and after incubation with red cell stroma.

incubation with stroma suspensions yielded significant results (Fig. 5).

Incubation of normal blood with normal stroma or with cord blood stroma, and incubation of pernicious anemia blood with normal stroma did not affect the slope of the curves for log per cent unaltered hemoglobin. But when normal adult blood was incubated with stroma from pernicious anemia blood, a kinked curve similar to those found in untreated pernicious anemia blood, was obtained.

DISCUSSION

When alkali is added to hemoglobin solutions, alkali hematin is formed. This is a monomolecular reaction, and when pure samples of fetal blood (cord blood) or normal adult blood are used, the values for log per cent unaltered hemoglobin plotted against time fall on a straight line. The slopes of the curves differ, indicating that the reaction proceeds more slowly where fetal blood is concerned.

Curves obtained from samples of mixtures of fetal and normal hemoglobin are kinked, the slope of the first part of the curves corresponding to the slope of adult hemoglobin, while the latter part of the curve has a slope similar to that of fetal blood. It is possible, by analysis of these curves, to obtain a reasonably accurate estimate of the amount of fetal hemoglobin present in the sample.

Similar kinked curves have been demonstrated in patients with sickle cell anemia and Cooley's anemia, as well as in other diseases where abnormal hemoglobin is known to be present. (Beaven & White, 1951; Liquori, 1951; Roche & Derrien, 1953; Singer Chernoff & Singer, 1951. When the presence of a pathological hemoglobin is suggested by the alkali denaturation method, it is usually possible to identify the pathological hemoglobin also by other methods. The finding of an abnormal alkali denaturation curve does not imply that the pathological hemoglobin is identical with foetal hemoglobin in all respects (White, Delory & Israel, 1950).

We have found an abnormal alkali denaturation curve to be a common feature in cases of pernicious and related anemias. But we have been unable to identify a supposed pathological hemoglobin by other methods, and our experiments seem to indicate that the abnormal curves are connected in some way with the stroma of the red cells, presumably with the stroma of the macrocytes.

While it is generally held that the demonstration of a kinked curve for log per cent unaltered hemoglobin indicates the presence of two different types of hemoglobin, Baar & Hickmans in 1941 suggested that the kinked curve might be due to an inhibitory action of the reaction products, and to absorbtion of CO_2. Such a possibility in cases of pernicious anemia must receive serious consideration in the light of work by Lajhta (1950) and Thompson (1950), who both have demonstrated the presence of substances with an inhibitory action on red cell maturation in pernicious anemia serum.

Fig. 6 may explain our findings:

At the top of the diagram is plotted the log per cent unaltered hemoglobin curve from the case of pernicious anemia in Fig. 1. The corresponding curve for optical density is plotted below.

In alternative 1 (left part of diagram) it is assumed that the sample contains two types of hemoglobin. The amount of "fetal hemoglobin" is estimated by extrapolating the last part of the log curve. By simple calculation, the corresponding optical density curves for the supposed fraction of "fetal" and "adult" hemoglobin are obtained (lower diagram left).

But another explanation of the kinked curve is equally possible (Fig. 6 — alternative 2).

If one assumes that the sample contains normal adult hemoglobin only, as suggested by our experiment 3, the first part of the log curve should be extrapolated. This will correspond to an optical density curve as indicated by the stippled line in the lower diagram right. To explain the optical density curve which actually was observed, it becomes necessary to imply an inhibitory action as indicated in the diagram. That some sort of inhibitory action is at work is strongly suggested by our experiment 4. The abnormal alkali denaturation curve in pernicious and allied anemias is only seen when macrocytes are present, and we suggest that these cells carry a stroma which in some way retards the alkali denaturation reaction. This suggestion is supported by the fact that the erythrocyte stroma and membrane is different in pernicious anemia from that of normal blood (Lemberg & Legge, 1949).

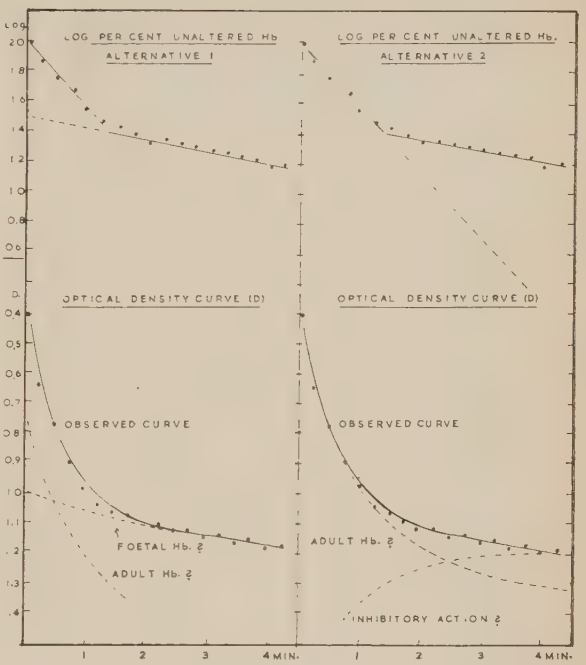

Fig. 6. Curves for log per cent unaltered hemoglobin and for optical density in a case of pernicious anemia.

Alternative 1 (left): Supposed presence of two types of hemoglobin.

Alternative 2 (right): Supposed presence of inhibitory substance.

Our experiments do not suggest that this inhibitory action is identical with, or in any way related to, the inhibitory substances which have been demonstrated by Lajhta and Thompson.

SUMMARY

Blood from patients with pernicious and allied anemias yield alkali denaturation curves suggesting the presence of an abnormal type of hemoglobin, resembling fetal hemoglobin. It has, however, been impossible to substantiate the presence of a pathological hemoglobin in these patients by other methods. Experiments are reported which suggest that the abnormal alkali denaturation curves found in cases of pernicious and allied anemias are due to substances in the red cell stroma which retard the reaction. It is suggested that this abnormal stroma is present in the macrocytes.

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Scandinav. J. Clin. & Lab. Investigation 168—169, 8, 1956.

ON THE CONWAY MICRODIFFUSION TECHNIQUE IN ROUTINE WORK

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(Received for publication January 3, 1956)

In our laboratory total CO_2 and urea determinations are made with the methods described by Conway (1950). The procedures which we have adopted are essentially as described, but some minor, convenient alterations in remedies will be described together with a very practical device for magnetic stirring in routine titrations.

Lids. For units No. 1 rectangular plates (85 × 70 × 3 mm) of clear glass with one hole 25 mm from one end are used. By pushing this lid to and fro, access to both chambers of the unit can be obtained through the same hole. This again is closed with a small block of glass.

Smears. A water-soluble substance is preferable. A mixture of glycerol and 2 N hydrochloric acid, 9:1 v/v for CO_2 analysis, and glycerol-water, 9:1 v/v, saturated with potassium carbonate for urea determinations are very satisfactory. They are easily applied to the lids with a brush, or better with a small piece of rubber foam, as big as the tip of a finger, partially pressed up in a piece of glass tube, this serving as a handle. When the smeared lids are placed firmly on the unit and then left alone, a perfect sealing is obtained within a minute.

Burettes. The type described by Conway (1950, p. 47, Fig. 14) has been adopted, but we have omitted the pressure-regulating tube (F), as it has been unnecessary, provided the capillary of the delivery tube is narrow enough. Two burettes, each with a capacity of 500 μl and divided in μl, are used. They are mounted on the same plate (Fig. 1). This arrangement has proved more convenient than the use of a burette of the Harman-Webster type (Conway, 1950, p. 49, Fig. 15).

Magnetic stirring device. (Fig. 2). This has been designed and made in collaboration with Mr. H. Fallesen, Institute of Physical Chemistry, and has proved very valuable in routine titrations. It consists of a stationary part, the frame, which can be fixed by screws under the table top. The motor is placed on the frame by means of a hinge so that the shaft of the motor leans against the movable wheel (Fig. 2, B) by the weight of the motor. The movable part consists of a metal house (diameter 75 mm), which can be moved through a hole in the table top, and in this house the magnet is hidden, mounted on the same axle as the wheel B. The base of the metal house rests on, and is operated up and down by the eccentric (Fig. 2, E), which again is operated with a handle at the edge of the table (Fig. 1, D). When the magnet house is in its "down" position, its upper surface is flush with the surface of the table. In its "up" position it is elevated 25 mm, and the tip of the burette will just be dipped in the solution to be titrated. The dimensions of the shaft of the motor and the movable wheel are chosen so that the magnet will rotate with a convenient speed. A variable speed control has proved unnecessary.

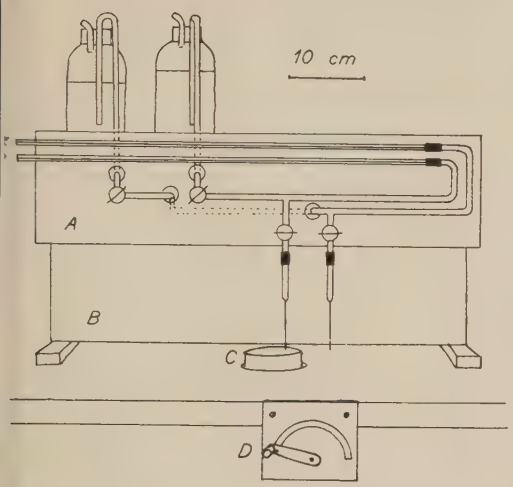

Fig. 1. *Arrangement for titration.* a and b are the calibrated capillary tubes of two Conway burettes, mounted on the same plate A, which is placed 10 cm in front of the box B with the two flasks. C is the magnet house of the stirrer, which is moved up and down by the handle D.

Notes on CO_2 determinations. Generally, 1 ml of sample is used in connection with unit No.1. The 1 ml volumes of 0.1 N Ba $(OH)_2$-indicator solution and of the sample are measured with ordinary Carlsberg constriction pipettes through the hole in the lid. The pipettes are operated by mouth with an absorber inserted to avoid complications from CO_2 in expired air. The absorber can be placed on the floor, for convenience. The use of pipettes with two constrictions has proved of no advantage when this technique is used, but they are indispensable when only 0.1 ml of sample is used in unit No. 2. Titration must be performed through the hole in the lid. 0.2 N hydrochloric acid is used.

Notes on urea determinations. Generally, only 0.1 ml of sample is used, as the whole range of values occurring for blood urea can then be covered with one filling of the burette, and the accuracy is still satisfactory. The saturated potassium carbonate solution

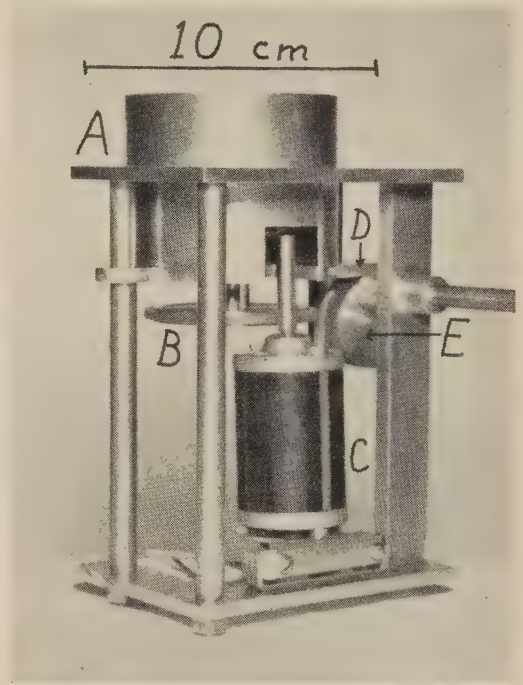

Fig. 2. *Magnetic stirring device.* The magnet, hidden in the house A, is mounted on the axle of the wheel B. The motor C leans against B, which slides along the shaft of C, when A is moved up and down by means of the eccentric E.

is introduced through the lid. 2 hours were found necessary for quantitative diffusion of the high values at room temperature. The lid is removed before titration of the boric acid solution, which is performed with 0.02 N hydrochloric acid.

Accuracy. The metods of Conway mentioned above are very satisfactory in routine work. They are easy to handle and the accuracy is good. In 1 ml serum, total CO_2 is easily determined with a standard deviation of 0.5 per cent. In 0.1 ml serum, urea is determined with a standard deviation of about 2 per cent for normal values and less for elevated values.

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A MICROMETHOD FOR TITRATION OF NON-SPECIFIC COLD-AGGLUTININS («AUTO-AGGLUTININS»)

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(Received for publication January 19, 1956)

In 1950 the author published a modification of the method for titration of cold-agglutinins in blood mixed with sodium citrate solution in the proportions used for the determination of the sedimentation rate (Trönnberg, 1950. The purpose of this paper is to describe a modification to test for cold-agglutinins in blood used in the *micro*-method for sedimentation rate measurement. This modification is easy to carry out and requires a minimum of outfit.

The principle of the method is a follows: Following the determination of the sedimentation rate, the plasma and erythrocytes are separated by centrifugation. The plasma is decanted and diluted with physiological saline in the usual geometrical series. The autogenous erythrocytes are diluted (without washing) with saline and aliqouts added to the plasma dilutions. Refrigeration and reading of results are similar to the standard method.

The principle advantage of this method is its uniformity. Whereas in earlier methods, 0-erythrocytes from different sources showed different degrees of sensitivity to cold-agglutinins, only autogenous erythrocytes are used. The fact that heterologous erythrocytes are not necessary also allows the procedure to be carried out more easily and in the smallest laboratories. Sources of error in the form of Rh-agglutinins, anti-0 isoagglutinins and other irregular agglutinins such as anti-M and anti-P are excluded. Agglutination due to bacterial growth (Thomsen, 1927) is also obviated if the test is carried out soon after taking of the sample. Therefore it is unnecessary to check for reversibility of cold-agglutination by warming. Finally, the method may be of value because of sparing of blood, especially in pediatric practice, for which it was originally devised.

The method, which is decribed below, is in principal the same as the previously described macro-method. However, in the present modification the blood is taken with capillary pipettes and citrated as in the micro-sedimentation rate method described by Ström (1933). Since the amount of plasma present is small, it is necessary to start the titration at a higher dilution than used in the macro-method.

METHOD

1. With the aid of three 0.125 ml pipettes (as described by Ström 1933) four volumes of blood are mixed with one volume of 3.8 per cent sodium citrate solution. After determining the sedimentation rate, the blood is centrifuged in a graduated centrifuge tube. The plasma is decanted by use of a screw-suction apparatus.
2. To each of five test tubes (approximately 55×10 mm) is added 0.3 ml physiological saline.
3. 0.1 ml citrated plasma is added to the first tube, mixed with the saline, and 0.3 ml of this mixture transferred to the next tube, and so on. In this way the last tube contains 0.6 ml of a 1 : 64 dilution of plasma in saline. This tube is stoppered and set aside so that if necessary higher dilutions may be titrated the following day.
4. The erythrocytes remaining in the centrifuge tube (about 0.15 ml) are diluted to a volume of 15 ml with saline. After mixing, 10 ml are removed and discarded. The remaining 5 ml are diluted with an equal volume of saline

resulting in a 10 ml volume of approximately 0.5 per cent suspension of erythrocytes.

5. Of the erythrocyte suspension 0.1 ml is added to the first tube and 0.3 ml to each of the three others. The tubes are then refrigerated for 8—24 hours at + 2° to + 5° C.
6. Results of agglutination in each tube are graded according to the method of Hedlund, Laurell & Löfström (1945).

5 +: The agglutinated erythrocytes have formed a disc, which does not break up when the tube is shaken.

4 +: The disc breaks up into large fragments on shaking.

3 +: The disc breaks up into macroscopic fragments on shaking.

2 +: The disc breaks up into submacroscopic fragments (but easily seen with a magnifying glass) on shaking.

1 +: The disc breaks up into very small fragments on shaking (observable only with a magnifying glass).

The limit for positive reactions is the tube that shows 3 +. It is important that the samples not be allowed to warm up during the time of reading. It is also important to remember that the first tube contains only 0.2 ml of a 1:8 dilution (compared with 0.6 ml of a 1:4 dilution in the first tube with the macro-method). This makes the reading in this tube somewhat uncertain.

RESULTS

The method described above has been evaluated simultaneously with the one previously described (Trönnberg, 1950) in 31 cases. 15 of these showed a titer higher than 1:16, 7 less than 1:16 and 19 completely negative with the macro-technique. The two methods were in very close agreement, the difference between the two techniques never exceeding one tube.

SUMMARY

A modification of previously described, simplified method for titration of non specific coldagglutinins has been presented, which utilizies citrated blood drawn in capillary pipettes for micro-sedimentation rate determination. The method is believed to have special significance for the interpretation of cold-agglutinin reactions on different days from the same subject. This increased reliability is obtained by the use of autogenous erythrocytes instead of stock 0-erythrocytes.

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PAPER ELECTROPHORESIS OF PORPHOBILINOGEN

(Received for publication March 27, 1956)

In the course of studying porphyrin pigments by paper electrophoresis it was observed that porphobilinogen, the substance responsible for the positive Ehrlich aldehyde reaction in the urine in cases of acute porphyria, can also be detected by this method.

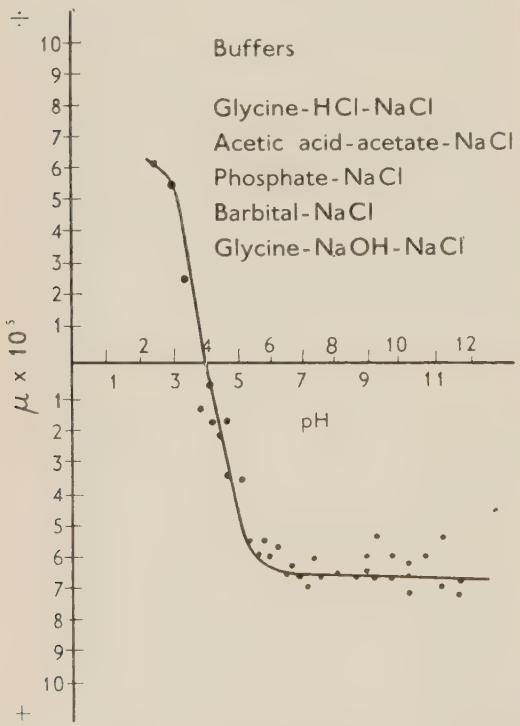

Fig. 1. Mobility values for porphobilinogen plotted against pH.

The present paper describes the method of separation of porphobilinogen and reports the results of an experiment which indicates the lines along which this test can be carried out.

METHODS

Repeated attempts by the writer to crystallise porphobilinogen were without success. The material used was prepared as follows: From the urine of a rabbit with experimental porphyria the porphobilinogen was precipitated with mercuric acetate according to Westall (1952). This was followed by decomposition with H_2S, which was removed with N_2. The solution was divided into parts and evaporated under reduced pressure, and the residues obtained were dissolved in the buffer to be used. The porphobilinogen concentration was 6 Vahlquist units per ml (Vahlquist, 1939). Then 0.04 ml of the solution was applied to Swedish Munktell paper 20/150 cut into strips 4 cm × 34 cm. At each run four strips were used with porphobilinogen solution and one with glucose as the measure of the electro-osmotic flow. The buffers were of various pH values (Fig. 1); all were of ionic strength 0.05 and a temperature of 20° C ± 1°. The current was 14—15 V/cm. After completed electrophoresis for one or two hours, the porphobilinogen was detected with Ehrlich aldehyde reagent (Partridge, 1942) and the glucose with aniline hydrogen phthalate.

In order to test the recovery a concentrated porphobilinogen solution was purified by continuous electrophoresis according to Grassmann (1953) using the buffer pH 3. With this purified solution a run was made at the same pH and under the above stated conditions. For localisation of the porphobilinogen two strips were sprayed with the aldehyde reagent. The corresponding areas from the other strips were cut out while wet and the porphobilinogen eluated for half an hour by shaking with 5 ml 1 M ammonium acetate. After suction of the solution through sinter 10 G/4 it was

made up to volume and 2.5 ml of a solution of 5 per cent p-dimethylaminobenzaldehyde in concentrated HCl was added. The color was measured within 3 minutes in the Beckman D. U. spectrophotometer at 555 mμ.

RESULTS

The mobility values of porphobilinogen at various pH levels are shown in Fig. 1. They correspond to those obtained by Tiselius electrophoresis by Waldenström and Vahlquist (1939). In the choice of pH for the run, pH 3 appeared to be optimal for the recovery test, since the porphyrins are absorbed into the paper at this pH and the substances which give a positive reaction with the Ehrlich reagent and which usually occur in the urine, such as tryptophane, urobilinogen and urea, did not interfere with porphobilinogen at this pH. The measurement as carried out under the above mentioned optimum conditions gave a recovery of 100 ± 5 per cent.

Department of Medical Chemistry,
University of Helsinki, Finland.

February 24, 1956. *Toa Heikel.*

SUMMARY

A rapid and simple method is described for the detection of porphobilinogen in urine by paper electrophoresis. The recovery of the substance purified by continuous electrophoresis was 100 ± 5 per cent.

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DEMONSTRATION OF A NEW PROTEIN FRACTION IN SERUM FROM THE HUMAN FETUS

(Received for publication April 5, 1956)

During an investigation of the serum proteins of human fetuses we have made an observation which seems worth of a short preliminary communication. Our purpose has been to study the electrophoretic pattern of fetal serum by paper electrophoresis carried out with the apparatus described by Dettker & Andurén (1954). The proteins were stained with Ponceau red.

Through cooperation of the obstetrical and gynecological department, S:t Erik's hospital, Stockholm we have so far been able to examine fetuses from 19 legal abortions. The fetuses ranged in length from 10 cm to 26 cm. Blood specimens were taken by heart puncture immediately after the operation. Blood from the mothers was drawn from a cubital vein at the same time and the electrophoretic patterns of maternal and fetal sera were compared.

In all the fetal sera examined we have found in a considerable concentration a new protein fraction which in the paper strip is situated between the albumin and the α_1-globulin (Fig. 1). In the maternal sera a corresponding fraction was not observed. The nature of this component, which does not seem to have been observed earlier in serum from human fetuses, is obscure. It has been suggested to us by Dr. K. Pedersen that the demonstrated protein may be the globulin fetuin earlier found by him (Pedersen, 1944, 1945) in fetal serum from different animals.

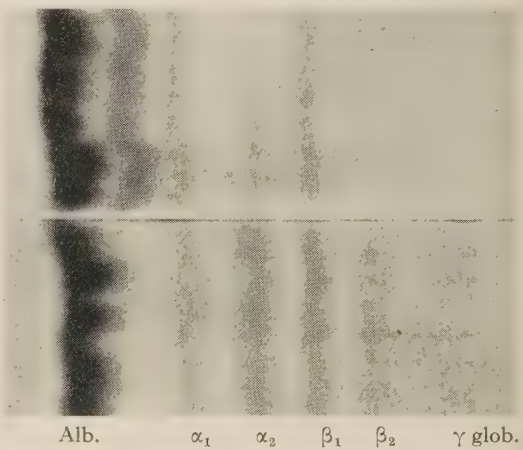

Fig. 1. Top: Fetal serum. Bottom: Maternal serum. Fetal length: 14 cm.

A study of the nature of the protein observed is in progress.

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SERUM ALDOLASE ACTIVITY IN PREICTERIC STAGE OF ACUTE HEPATITIS*

In recent years, the significance of serum aldolase determinations for the diagnosis of acute hepatitis has been emphasized by several authors. Aldolase is the enzyme responsible for the cleavage of fructose-diphosphate into two triosephosphate molecules. Bruns (1954), Sibley & Fleischer (1954) and Løken (to be published) have demonstrated that in early stages of acute hepatitis, the serum aldolase activity is increased several times, while in other liver diseases, such as cirrhosis, obstructive jaundice, cancer (primary or secondary), cholangitis and cystitis, the aldolase activity is either normal, or only moderately increased. As the hepatitis subsides, the serum aldolase activity rapidly decreases. In mild cases the normal level is usually reached within 6 to 14 days from the onset of icterus. In severe or chronic cases the raised aldolase level may continue for several weeks or even months. A typical course of progress is shown in Fig. 1, representing a moderately severe hepatitis in a 30 year old woman. The curve indicates that the increase of the aldolase activity starts somewhere in the preicteric stage of the disease.

During an epidemic a in small rural community in Norway the author succeeded in demonstrating in two patients that serum aldolase activity was increased in the preicteric stage of acute hepatitis.

The aldolase activity was determined according to the method of Sibley & Lehninger (1949) and expressed as extinction values of the color developed, obtained with 10 mm cuvettes in Uvispec spectrophotometer. (Normal values range from 0.050 to 0.185.)

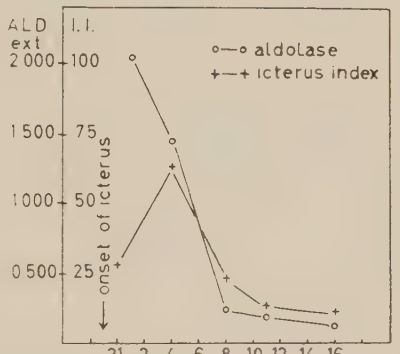

Fig. 1. A case of moderate hepatitis, observed from the second day after onset of icterus.
Ald. ext. = Aldolase activity expressed in terms of extinction values.
I.I. = Icterus index.

A large number of healthy persons belonging to families where one or more of the family members were suffering from hepatitis were examined. All observations on persons feeling completely well, and with no other signs of hepatitis, showed normal serum aldolase activity.

A 30 year old male was seen on March 2, when he felt quite well. The serum aldolase was normal. On March 3 he started to feel ill, with nausea. He went to bed on March 5. He was seen again on March 9, 6 days after the onset of initial symptoms. There was no visible jaundice, nor was there any brown

* Aided by a grant from A/S Freia Chokoladefabriks Fond

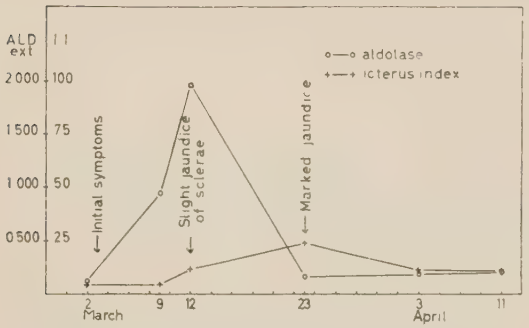

Fig. 2. A case of moderate hepatitis, observed from the day before onset of symptoms.
Ald. ext. = Aldolase activity expressed in terms of extinction values.
I.I. = Icterus index.

color of the urine. The blood test showed a marked increase in serum aldolase activity, and normal icterus index. On march 12 there was a slight icteric color visible in sclerae, with icterus index 11.5. Aldolase activity 1.955. On March 23 the aldolase activity was normal, while icterus index was increased to 25. His chart is given in Fig. 2.

The second case was a female about 42 years of age, who was seen on Feb. 24. She had taken ill on the previous night with nausea. The serum aldolase was significantly above the normal level (ext. value 0.390). and continued to increase for the next five days, reaching 2.020 (15 to 20 times normal value). From the 12th day serum aldolase activity was again normal. Icterus index was significantly increased from the 7th day (March 2), and reached its maximum on the 11th day from the onset of initial symptoms. Her chart is given in Fig. 3.

A third case concerns a 35 year old woman who was seen 5 days after the onset of intial symptoms, which were nausea, pains in legs and hips, and slight fever. The serum aldolase level was 1.480 expressed in terms of extinction values obtained. Icterus index

University Clinical Laboratory,
Dept. of Biochemistry,
Rikshospitalet.
April 20, 1956.
Oslo, Norway.

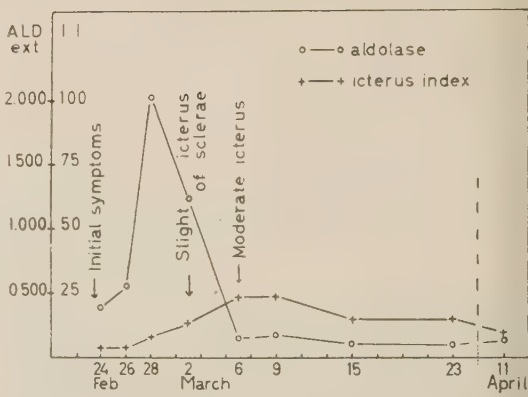

Fig. 3. A case of moderate hepatitis, observed from the day of onset of symptoms.
Ald. ext. = Aldolase activity expressed in terms of extinction values.
I.I. = Icterus index.

7.5. 11 days later the serum aldolase was normal, and so was the icterus index, (5.5). She did not develop any visible icterus, and recovered within a few days. The case is considered an anicteric hepatitis.

From the present observations it seems probable that determination of serum aldolase activity may be of importance in the early diagnosis of acute hepatitis.

ACKNOWLEDGEMENT

The author is greatly indebted to Dr. Thor Bjerkedal of the institute of Hygiene, University of Oslo, who has kindly performed the Icterus Index tests, and been of great help in collecting the blood samples.

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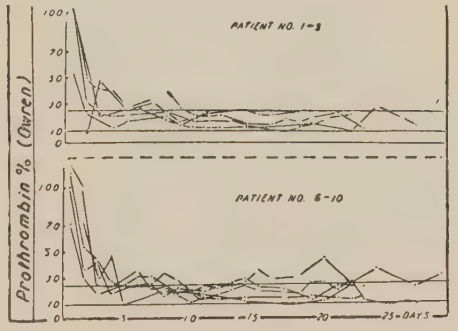

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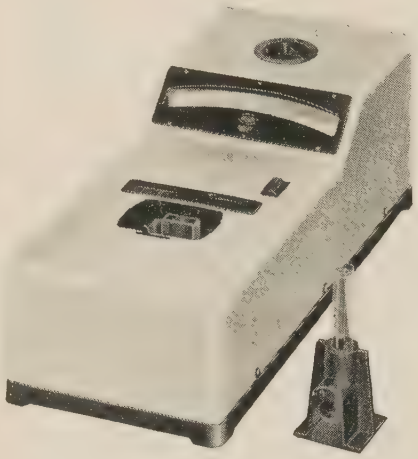

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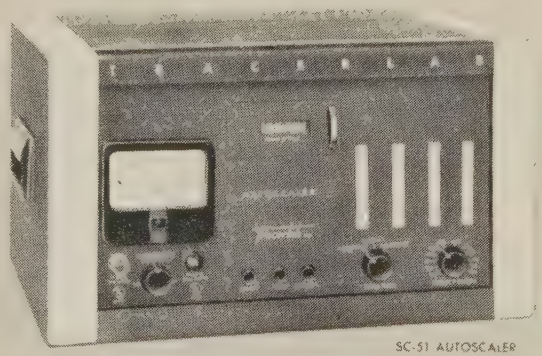

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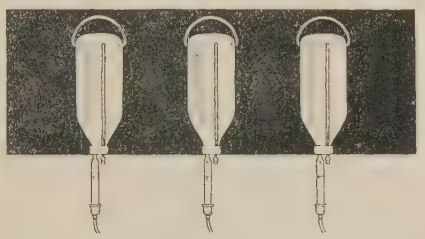

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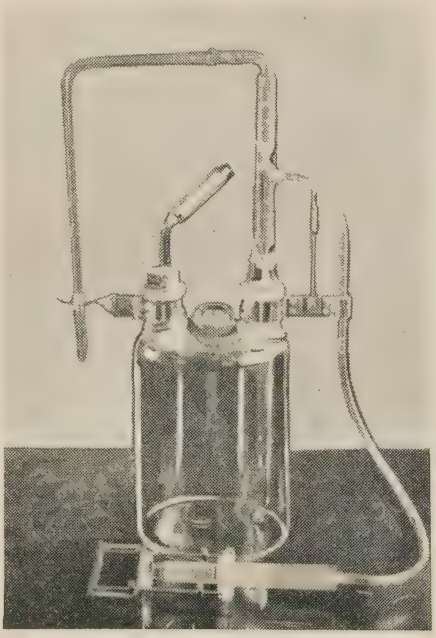

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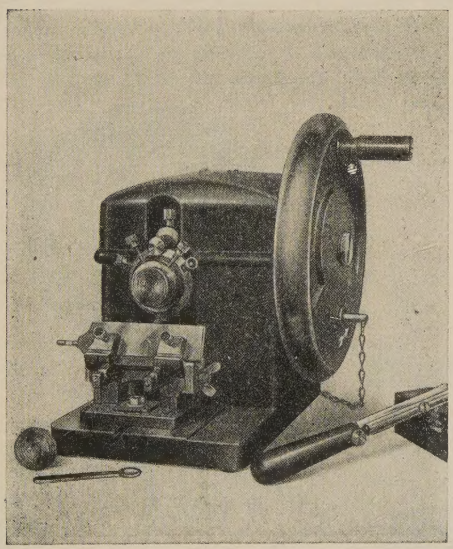

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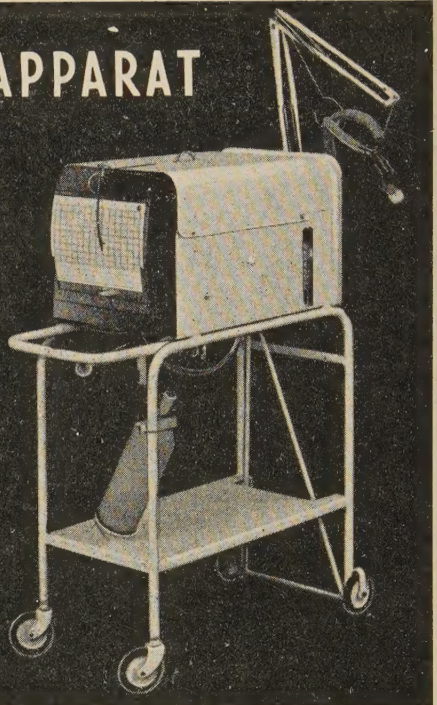

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